

Imperial College London
Department of Chemical Engineering

**The Humanisation and Generation of Antibody
Fragments against Oxidised Low Density
Lipoprotein for the Optical Molecular Imaging of
Atherosclerosis**

Samata Shrestha Pandey

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I hereby declare that this thesis and the work reported herein was composed by and originated entirely from me. Information derived from the published and unpublished work of others has been acknowledged in the text and references are given in the list of sources.

Samata Pandey (2021)

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Abstract

Atherosclerosis and its major adverse cardiovascular events remain a leading cause of mortality and morbidity globally. Despite advances in diagnostics and treatment, a key barrier to reducing its consequences is the difficulty in predicting and identifying those plaques which are most vulnerable to rupture. Monoclonal antibodies provide a unique opportunity in tackling this gap due to their natural high specificity for an epitope; making them promising imaging agents for the molecular imaging of atherosclerosis. In this effort, single chain variable fragments (scFv) and fragment antigen binding (Fab) of a characterised murine monoclonal antibody, LO1, have been generated for the molecular imaging of oxidised low density lipoprotein. These small size of these antibody fragments may optimise tissue penetrance and kinetics for molecular imaging, generating increased signal to noise ratio and greater targeting. In a translational effort, a humanised Fab was generated to minimise potential immunogenicity. The rationale design and expression of the humanisation and antibody fragment generation is presented. Near-infrared labelling of the humanised Fab allowed non-invasive imaging of atherosclerosis in a mouse model using fluorescence molecular tomography (FMT). Results revealed the humanised Fab successfully distinguishes atherosclerotic animals from wild-type mice. Multiplexed imaging with fluorescent probes targeting matrix metalloproteinases and integrin $\alpha 5/\beta 3$ discovered the humanised Fab was able to distinguish between diseased and non-diseased animals with the greatest confidence. Overall, this thesis demonstrates the utility of antibody fragments for molecular imaging.

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List of Abbreviations

ddH₂O Double-distilled water

DAPI 4',6-diamidino-2-phenylindole

AID Activation-induced cytidine deaminase

ABS Antigen binding site

ApoB Apolipoprotein B

ApoE Apolipoprotein E

ATHEROMA Atorvastatin Therapy: Effects on Reduction of Macrophage Activity

ABC ATP-binding cassette

BSA Bovine serum albumin

CIP Calf Intestinal Alkaline Phosphatase

CV Column volume

CDR Complementarity determining region

CT Computed tomography

CHO Chinese hamster ovary

DICOM Digital Imaging and Communications in Medicine

D Diversity

DPBS Dulbecco's Phosphate Buffered Saline

ER Endoplasmic reticulum

EMA European Medical Agency

FILM Facility for Imaging by Light Microscopy, Imperial College

FMT Fluorescence Molecular Tomography

FDA Food and Drug Administration

HC Heavy chain

Ig Immunoglobulin

ICG Indocyanine green

IDL Intermediate density lipoprotein

IMGT International ImMunoGeneTics Information System

IVIS In Vivo Imaging System

IVUS Intravascular ultrasound

J Joining

LC Light chain

LDLR Low density lipoprotein receptor

LDL Low density lipoprotein

MRI Magnetic resonance imaging

MDA Malondialdehyde

MOE Molecular Operating Environment

NASH Nonalcoholic steatohepatitis

NIR Near infrared-red

NLRP3 NLR Family Pyrin Domain Containing 3

OCT Optical coherence tomography

oxLDL Oxidised LDL

PBST PBS-Tween20

PCR Polymerase Chain Reaction

PTM Post-translational modification

PCSK9 Pro-protein convertase subtilisin/kexin type 9

RSS Recombination signal sequence

SHM Somatic hypermutation

SEM Standard error of the mean

SOC Super Optimal Broth with Catabolite repression

TCFA Thin-cap fibroatheroma

TNF Tumor necrosis factor

USPIO Ultrasmall superparamagnetic iron oxide

V Variable

WT Wild-type

WHO World Health Organisation

Amino acids

One letter code	Three letter code	Amino acid
A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine

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Chapter 1

Introduction

Despite the pivotal introduction of statins in the late 1980s cardiovascular diseases remain the leading cause of mortality globally and a significant contributor to a reduced quality of life for those affected (Roth et al. 2018, Kyu et al. 2018). Acute coronary events, such as myocardial infarction (MI), arise from the rupture of atherosclerotic plaques in coronary arteries. The rupture exposes the thrombogenic interior to the luminal blood, resulting in the formation of a clot and subsequent acute event. Post-mortem studies have revealed that those who experience sudden cardiac death often have culprit plaques that are particularly large, occupying over 50% of the vascular cross-sectional area. Further characteristics that have been identified include having extensive plaques with necrotic cores, a thin fibrous cap, high levels of inflammation and/or neovascularisation (Virmani et al. 2006).

Current clinical practice relies on known risk factors to project the likelihood of an acute event such as age, lifestyle, circulating biomarkers and luminal stenosis. Our understanding of the disease has vastly improved whereby it is now understood that it is an insidious and self-perpetuating disease which may show no luminal stenosis prior to plaque rupture. Further to this, a third of patients who experience a major cardiovascular event (MACE) will represent with another within three years arising from either the initial lesion or a previously non-culprit lesion (Stone et al. 2011). The Clinical Outcomes Utilizing Revascularisation and Aggressive

Drug Evaluation (COURAGE) trial demonstrated that angioplasty and stent placement in patients with chronic stable coronary artery disease was not more effective than drug treatments as measured by the death rate and rate of myocardial infarction post-placement (Boden et al. 2007). This trial emphasises the importance of focus on plaque composition and predictors of vulnerability as the path to reducing the incidence of acute coronary events. There remains a fundamental gap in the clinical evaluation and treatment of atherosclerotic plaques to prevent mortality and a loss in quality of life.

The early detection and characterisation of plaques prior to their rupture is imperative in preventing fatal events and improving quality of life for those affected. Thus, this thesis aims to develop a reliable and objective approach to identify ‘vulnerable plaques’ in the hopes that the improvement in assessing the presence of these plaques may aid in patients receiving tailored treatment on the basis of their individual risk. A significant proportion of plaques responsible for MACE have defined characteristics, including elevated levels of oxidised low density lipoprotein (oxLDL). As such, the work presented herein focuses on malondialdehyde-LDL (MDA-LDL), a form of oxLDL identified to be present in plaques in both human and animal models of atherosclerosis (Amaki et al. 2004, Virmani et al. 2006, Ito et al. 2018).

Armed with a defined target for identifying high risk plaques, three remaining components are required: an imaging agent, a method of imaging this agent and a model in which to test its efficacy.

With regards to the imaging agent, the scientific community has harnessed the specific targeting abilities of monoclonal antibodies (mAbs) to market over 100 mAb-based drugs for use in a broad range of diseases (Kaplon & Reichert 2021). The isolation and identification of a murine antibody, LO1, which targets MDA-LDL provides a reagent for the detection of vulnerable plaques. Murine monoclonal antibodies are relatively easy to produce due to robust manufacturing processes but are severely restricted for therapeutic use due to their immunogenicity. Whilst fully human antibodies are preferred for clinical use, the cost and difficulty in producing them has slowed their uptake within the pharmaceutical industry. Genetic

engineering has opened a compromise by enabling the generation of humanised antibodies, which combine the antigen binding sites from murine antibodies with human antibody frameworks (Winter 1991) allowing a vastly improved *in vivo* tolerability (Harding et al. 2010).

Current atherosclerotic imaging techniques are able to obtain morphologic and functional plaque information, however, they often employ a high radiation dose which may not be acceptable for regular clinical use. Other techniques suffer from spatio-temporal resolution problems in small vessels such as the coronaries. An attractive non-invasive imaging method for pre-clinical studies is fluorescence molecular tomography (FMT). Near-infrared fluorescence imaging (NIRF) provides an adaptable alternative to currently available imaging techniques. This method is amenable to allow molecular imaging, whereby targeted agents can detect, visualise and quantify molecular processes *in vivo*.

Finally, a disease model is needed for testing the imaging agent *in situ*. The low density lipoprotein receptor deficient (LDLR^{-/-}) mouse model has a mutation in the receptor rendering it unable to endocytose LDL (Ishibashi et al. 1993). Similarly in humans, LDLR is not able to recognise the pathogenic oxLDL. Therefore, this mouse model sufficiently resembles the human plasma lipoprotein profile giving it the potential to give translatable results to humans (Khan et al. 2018).

This thesis ties together the three fields of atherosclerosis, molecular imaging and antibody engineering to advance pre-clinical studies of atherosclerosis. Chapter 2 provides a general introduction to antibodies, their modular structure and how this has contributed to the importance of monoclonal antibodies as diagnostics and therapeutics. This is followed by an introduction into atherosclerosis as a disease, its pathobiology and the role of oxLDL in its formation. A comparison of current molecular imaging techniques for targeted imaging of such diseases is also discussed, with a focus on near-infrared fluorescence imaging. Finally, the chapter will introduce LO1, a murine monoclonal antibody, that is the focus of the study. Specifically the project set out to create, humanise and test LO1 antibody fragments with a view to using these for diagnostic imaging in cardiovascular disease. In Chapter 3, a list of experimental

methods employed throughout the research in this thesis is described. The research commences in Chapter 4, with the *in silico* humanisation of LO1 and its expression and *in vitro* and *ex vivo* characterisation in a Fab format. Chapter 5 demonstrates the utilisation of the humanised Fab fragment within the LDLR^{-/-} model. It uses *ex vivo* imaging as validation of specific signal in the aortic arch. In a similar approach to Chapter 4, Chapter 6 presents the design of LO1 as a single chain variable fragment (scFv) and diabody fragment and its *in vitro* and *ex vivo* characterisation. Finally, the thesis closes in Chapter 7 by providing a final conclusion and discussion on the use of antibody fragments and their humanised equivalents for molecular imaging and the limitations to this approach. It sets out future studies that can be carried out and some novel tangential techniques available.

Chapter 2

Literature review

2.1 History of antibodies as biological agents

The existence of antibodies was proposed over a century ago, when Emil von Behring and Shibasaburo Kitasato demonstrated that animals suffering from diphtheria could be cured through the transfer of serum from animals immunised against it (von Behring 1890). They were subsequently awarded the Nobel Prize in 1901 for this discovery.

A collaborator of von Behring, Paul Ehrlich, quickly followed this up with his “side-chain theory” in which he hypothesised that antibodies bind to antigens through special chemical structures called side-chains (receptors). He received the Nobel Prize in 1908 for this work (*NobelPrize.org* n.d.a). As the field grew, a key hindrance was the inability to generate, isolate and purify antibodies with a known specificity. The solution came in 1975 from a collaboration between Georges Köhler and César Milstein and the invention of the hybridoma technique, in which plasma cells are fused with myeloma cells. Plasma cells produce antibodies of a single specificity but have a short lifespan, while myeloma cells can grow indefinitely in culture. In their paper, Köhler and Milstein correctly predicted that their invention “...could be valuable for medical and industrial use” (Köhler & Milstein 1975). They later received the Nobel Prize in 1984. Their pioneering technique has been widely recognised as the start of the modern era

of antibody discovery and research. Not long after, in 1986, the first therapeutic monoclonal antibody, muromonab-CD3 (Orthoclone), was approved by the Food and Drug Administration (FDA) for the prevention of kidney transplant rejection (Goldstein & G. 1985). Since then, antibodies have become major biological drugs with over 80 antibody therapeutics marketed between 1986-2019 (Kaplon et al. 2020).

2.2 Antibody structure and function

Immunoglobulins (Igs), the molecular basis of antibodies, are antigen-specific glycoproteins secreted by plasma cells and B-lymphocytes. They play an important role in the immune system and either arise spontaneously or in response to pathogens (Binder & Silverman 2005). In humans, antibodies are classified into five families (isotypes) on the basis of their heavy chains: α (IgA), δ (IgD), ϵ (IgE), γ (IgG) and μ (IgM). The different isotypes differ from one another in their biological function, location and macromolecular structure. IgM forms a pentamer and is part of the immune system's innate response, the first line of defence. In contrast, IgG is a monomer, the most prevalent isotype in serum and is responsible for protecting against most pathogens, forming part of our adaptive immune system. IgE, a monomer, binds to allergens and triggers histamine release. IgA forms a dimer and is found in mucosal areas and prevents colonisation by pathogens. It is believed that monomer IgD plays a role in mucosal immunity, although this remains to be established (Übelhart et al. 2015, Choi et al. 2017). The specific functions and properties of antibodies are dependent on their isotype. For example, a humanised antibody was effective prophylactically against yellow fever as an IgG but not in the IgM form (Thibodeaux et al. 2012).

In 1958 and 1961, Rodney Porter and Gerald Edelman separately elucidated the antibody structures we now routinely use. They were jointly awarded the Nobel Prize in 1972 for their work on unravelling the covalent structure of antibodies (*NobelPrize.org* n.d.b). They elucidated that an antibodies' covalent structure is comprised of a fragment crystallisable (Fc, otherwise known as constant region) and fragment antigen-binding (Fab), which are joined by disulphide

bonds at the hinge region. The Fab can be further divided into the constant region (CH1 and CL) and the fragment variable (Fv; VH and VL). The Fc portion is responsible for inducing antibody-dependent cell mediated cytotoxicity (ADCC) and has been shown to activate the complement system and induce complement-dependent cytotoxicity (CDC). The Fv portion is more variable in sequence and contains the antigen binding site (ABS), which is responsible for interacting with the antigen. The ABS is composed of six ‘hypervariable’ loops, known as the complementarity determining regions (CDR) as shown in Figure 2.1.

Each domain in the antibody is composed of β -strands which pack together to form two β -sheets. These two sheets are joint together by a disulphide bridge forming a β barrel. This distinctive structure is known as the immunoglobulin fold and can be found in B-cell receptors, T-cell receptors, and other members of the immunoglobulin superfamily (Buck 1992).

To date, IgG is the isotype that has been most widely studied structurally (Figure 2.1). It consists of two pairs of identical heavy (HC) and light chains (LC), where the LC can be a κ or λ and the HC is γ . Each LC and HC pair forms an “arm” of the antibody. A HC consists of one variable domain (VH) and three constant domains (CH 1-3), while the LC consists of one variable domain (VL) and one constant domain (CL). The HC and LC each provide three of the hypervariable loops which form the ABS. The hinge region is located between the first and second constant domains (CH1-CH2). The exact composition of the hinge region is dependent on the subtype of IgG (Vidarsson et al. 2014).

Despite the ability of antibodies to bind specific antigens, there is a remarkable amount of structural similarity between antibodies. Based on structural information, five out of the six CDRs can be clustered into “canonical classes” (Chothia & Lesk 1987, North et al. 2011). Canonical structures are standard conformations that the main-chain and side-chains of CDR residues adopt based on the location of key residues and length of the loop (Chothia & Lesk 1987, Al-Lazikani et al. 1997, North et al. 2011). Thus, two different antibodies with the same loop length and same key residues will adopt the same structure, despite other differences in CDR sequence. This understanding of canonical structures has become the underpinning of

the prediction of engineered antibody structures for their humanisation or improvements in biophysical properties. A key challenge to this is that of the six CDRs. CDR-H3 is the longest and most variable CDR making its' structure the hardest to predict (Glanville et al. 2009, Almagro et al. 2014). However, this same CDR is often a key player in antigen recognition (Bujotzek et al. 2016, Barrios et al. 2004).

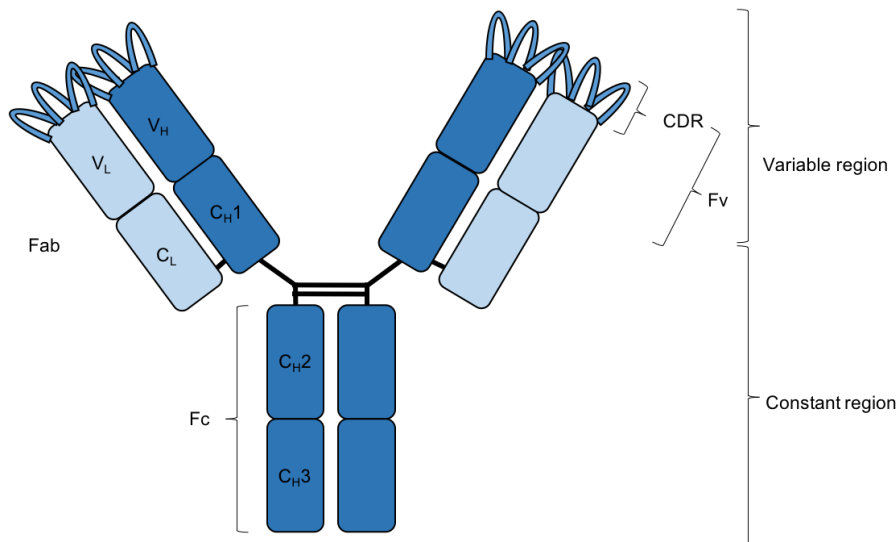


Figure 2.1: Structure of IgG molecule. Schematic diagram showing the antibody structure of Immunoglobulin G molecule. The fragment crystallisable (Fc) is made of two domains: CH3 and CH2. The Fab domain is made of four domains, two from the light chain (light blue): CL and VL and two from the heavy chain (dark blue): VH and CH1. The Fab also contains the fragment variable (Fv) which is the most variable part of the antibody with part of that being the complementarity determining regions (CDR) which form the antigen binding site.

2.2.1 Numbering and CDR Definitions

Wu & Kabat (1970) presented seminal work where they analysed the sequences of antibodies and proposed a somatic theory of antibody diversity. Without structural information, they defined the CDR of an antibody as the most variable regions in terms of sequence. As X-ray crystallographic structures became available it was evident that the CDRs are not limited to the apical loops only, but can extend along the β -strands themselves (Poljak et al. 1973, Schiffer et al. 1973). This additional structural information led to new definitions such as those proposed by Chothia and Lesk, which incorporated structural differences into the CDR definition (Chothia & Lesk 1987). The International ImMunoGeneTics Information System (IMGT) produced a

general definition of the CDR which is independent of the type of receptor (antibody, B Cell Receptor or T-cell receptor), but relies on the folding pattern (Giudicelli et al. 2004). The Kabat, Chothia and IMGT numbering systems are the most commonly used, with many other numbering systems now established. The key differences in these three numbering systems lies in their definitions of the different CDRs as demonstrated in Table 2.1. In this thesis, Kabat definition is the primary numbering system used with other numbering systems being identified when used.

Table 2.1: CDR definitions of antibodies. For each CDR and naming scheme, the start and end residue of each loop is reported.

CDR	Kabat	Chothia	IMGT
L1	L24-L34	L24-L34	L27-L32
L2	L50-L56	L50-L56	L50-L51
L3	L89-L97	L89-L97	L89-L97
H1	H31-H35B	H26-H32	H26-H35B
H2	H50-H65	H52-H56	H51-H56
H3	H95-H102	H95-H102	H93-H102

2.2.2 Generation of antibody diversity

In the 1970s, debate was rife as to how antibody diversity was generated. The two central theories were the germline theory, where diverse proteins are encoded by different genes, and the somatic differentiation theory, where diversity results from variation occurring during replication. In 1976, molecular biologist Susumu Tonegawa uncovered the processes of somatic recombination and somatic hypermutation. In combination, these two processes allow the human naïve antibody repertoire to diversify to at least 10^{12} or even 10^{15} unique antibodies (Briney et al. 2019, Mak et al. 2014). It is for this substantial discovery that he was awarded the Nobel Prize for the genetic principle of antibody diversity (Hozumi & Tonegawa 1976). The processes of somatic recombination and somatic hypermutation occur within B cell lymphocytes, as part of immune adaptation.

Immunoglobulin genes

In a developing B cell, the variable domains of the HC and LC are encoded by exons which are physically distant from the exons encoding the constant domains. The variable exons are further subdivided into DNA fragments called gene segments. The HC has three gene segments: Variable (V), Diversity (D) and Joining (J) while the LC has only two as there is no D gene segment. In humans, the *Igh*, *Igκ* and *Igλ* loci are on chromosomes 14, 2 and 22 respectively (Mak et al. 2014). Allelic exclusion of the light chain ensures that only one LC is paired with HC.

Somatic recombination

Somatic recombination is the process whereby gene segments from the pool of genes are randomly rearranged to generate diversity. In the first stage, gene rearrangements generate a new HC (Figure 2.2). This is where a single D gene fuses to a single J gene. This is followed by the D gene fusing with the V gene forming the VDJ region. Each gene is flanked by either one or two recombination signal sequences (RSS) consisting of a heptamer-spacer-nonamer, where the spacer can either be 12 or 23 base pairs (Bassing et al. 2000). Molecular processes such as the 12/23 rule, where a RSS with a 12 base pair spacer can only recombine with a RSS with a 23 base pair spacer ensures that correct recombination occurs. The failure of this combinatorial assembly has been implicated in conditions such as lymphoid neoplasms (Tsujimoto et al. 1985) and immunodeficiency disorders (Moshous et al. 2001).

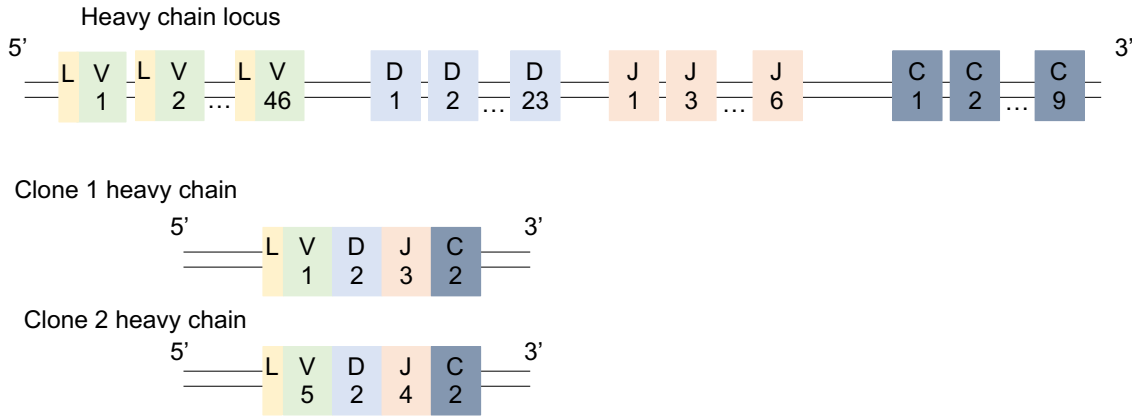


Figure 2.2: Schematic diagram of somatic recombination. The process of somatic recombination generates multiple variants from the heavy gene locus. V, D, J gene segments are located upstream of the constant region. Each V gene is followed by a leader sequence. Each gene segment is flanked by a RSS. This figure is representative of heavy chain recombination, as light chains do not have a D gene segment. V, variable. D, diversity. J, joining, RSS, recombination signal sequence.

Somatic hypermutation

The second process, somatic hypermutation (SHM) introduces random point mutations thereby further increasing diversity of Igs. Activation-induced cytidine deaminase (AID) is crucial for SHM occurrence and mutations in this enzyme gene can result in AID deficiency resulting in a lack of SHM and class switching of antibodies (Péron et al. 2007). Detrimental mutations which alter basic Ig structure are eliminated through a process of negative selection (Murphy & Weaver 2017). Conversely, mutations which increase the affinity of the Ig are favored and these B cells expanded at this stage. Analysis of CDR sequences have revealed that mutations have the highest frequency in the CDRs, while mutations in the constant or framework region are less frequent (Figure 2.3). Mutations occurring in the constant or framework regions are often silent or neutral mutations whereby the amino acid sequence is conserved (Glanville et al. 2009). A key resulting feature of SHM is the production of an Ig with increased affinity for the antigen, a process known as affinity maturation.

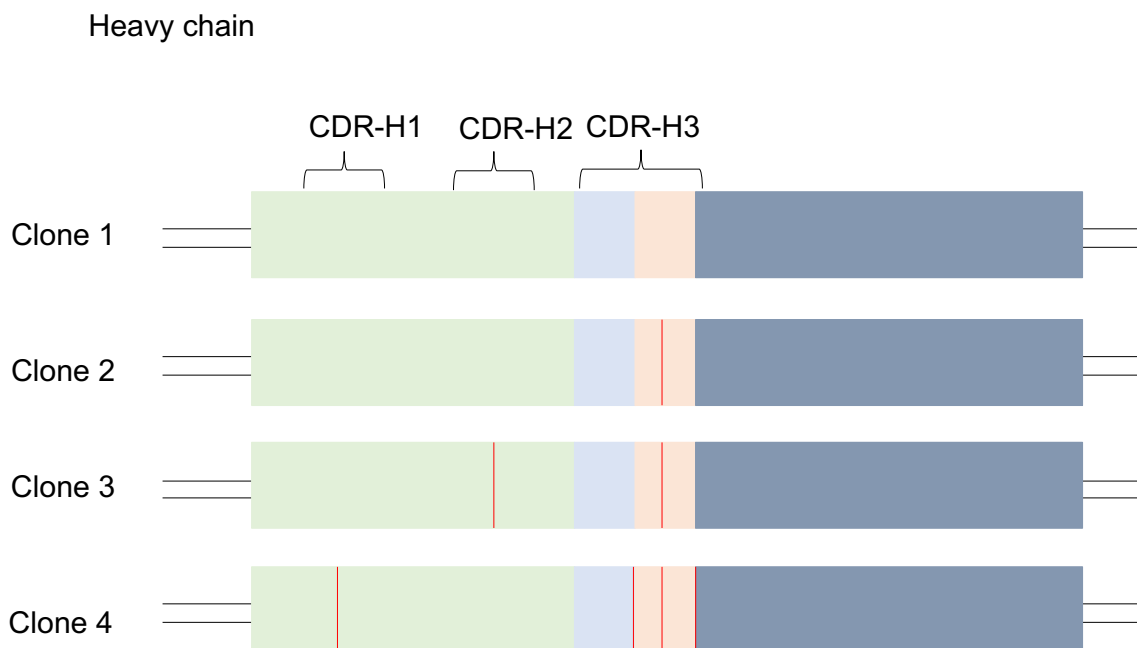


Figure 2.3: Schematic diagram of somatic hypermutation. Red lines identify regions of heavy chain hypermutations, which are most commonly found in the CDRs.

2.2.3 Pairing of light and heavy chains

The identification of the endoplasmic reticulum (ER) resident chaperone BiP and its seminal role in protein translocation has shed light into the processes involved in antibody folding and assembly (Haas & Wabl 1983). BiP-associating sequences are found primarily in β -strands that are necessary for interactions between the individual domains. BiP associates with the unfolded CH1 domain retaining it within the ER. Only once the LC is covalently added through a disulphide bond between the CL and CH1 domains can the CH1 domain fold and be secreted, thus preventing the release of HC dimers (Stoyle et al. 2017). Despite having BiP associating sequences, studies have revealed that LCs can fold and be secreted either as monomers (Dul et al. 1996) or dimers (Leitzgen et al. 1997), therefore acting independently of BiP (Hellman et al. 1999).

The intermolecular forces aiding the pairing of VH and VL domains have been highlighted in the framework regions. Ionic interactions between arginine and aspartic acid and paired glutamine residues strengthen the domain interactions. Conservation of aromatic residues are

important in forming the “packing” structure between the VH and VL domains (Chothia & Lesk 1987). Additionally, hydrophobic interactions between the domains account for strong association between domains (Hamel et al. 1984). While hydrogen bonding is important, the domains can remain associated without this interaction.

2.2.4 Prediction of antibody structure

Template-based modeling, also known as homology modeling, is based on the understanding that high sequence similarity also results in high structural similarity (Chothia & Lesk 1987). Using the sequence, a template is predicted from available structures and refined according to the amino acid sequence of the specific protein. Existing antibody modeling tools can predict the variable domain with an accuracy close to 1.0Å (Almagro et al. 2014), although CDR-H3 is poorly modelled with an average accuracy greater than 1.5Å. Specifically for antibodies, canonical structures for CDRs are widely accepted to follow a given loop structure, with the exception of CDR-H3 (Chothia & Lesk 1987).

In contrast, *de novo* prediction is necessary when a template does not exist and is predicted from the first principles of energy minimisation. This is computationally intensive and to date has been demonstrated to be effective only for proteins less than 100 amino acids in length (Maximova et al. 2016). Apart from individual loop prediction, *de novo* prediction is not widely utilised for antibody modeling as homology modeling already provides high accuracy.

2.2.5 Modular nature of antibodies

The modular structure of antibodies makes it particularly accessible to protein engineering. The inclusion of domains that are beneficial for the intended use can be incorporated, but those that are not can be excluded. Figure 2.4 shows the antibody fragments most relevant to this thesis. Table 2.3 shows antibody formats predominantly used for imaging.

Fab fragments are the oldest class of antibody fragments (Nelson 2010) and are one of the most widely used biomolecules as they have greater penetrance due to their smaller size while retaining specificity (Feige & Buchner 2014). They were first created by cleavage of a full antibody with the enzyme papain, which digests the peptide bond in the hinge region creating two monovalent Fab fragments (50kDa each) and one Fc fragment (50kDa) (Figure 2.4). A Fab' is generated from a full mAb by the digestion of pepsin and β -mercaptoethanol for disulphide bond reduction. Both Fab and Fab' fragments have reduced avidity compared to the full IgG due to their monovalency.

Huston et al. (1988) created the single-chain fragment variables (scFv, 25kDa) which is the smallest producible fragment with a complete ABS. They are engineered most commonly by the addition of a peptide linker joining the VH and VL chains, allowing the domains to associate by non-covalent interactions (Figure 2.4). The length of the linker and orientation of VH-VL segments can have profound effects on antibody-binding abilities. scFvs show clinical potential with several undergoing clinical trials (Laetsch et al. 2019).

For diagnostic purposes, a fragment without the Fc may be desirable due to unwanted Fc effector functions. Diabodies, Fabs, $F(ab')_2$ and scFvs may serve this purpose. The removal of the Fc will also reduce the size of the molecule allowing for its faster clearance which may be beneficial in certain circumstances. However, antibody fragments may continue to possess undesirable properties that can warrant further engineering. For example, Fv fragments are non-covalently associated heterodimers which may dissociate. In contrast, engineered stable Fv fragments have the addition of a flexible and hydrophilic linker between the two domains improving the stability for *in vivo* use (Bird et al. 1988, Huston et al. 1988). For therapeutic purposes, a full mAb may be desired due both to its effector functions and longer half-life.

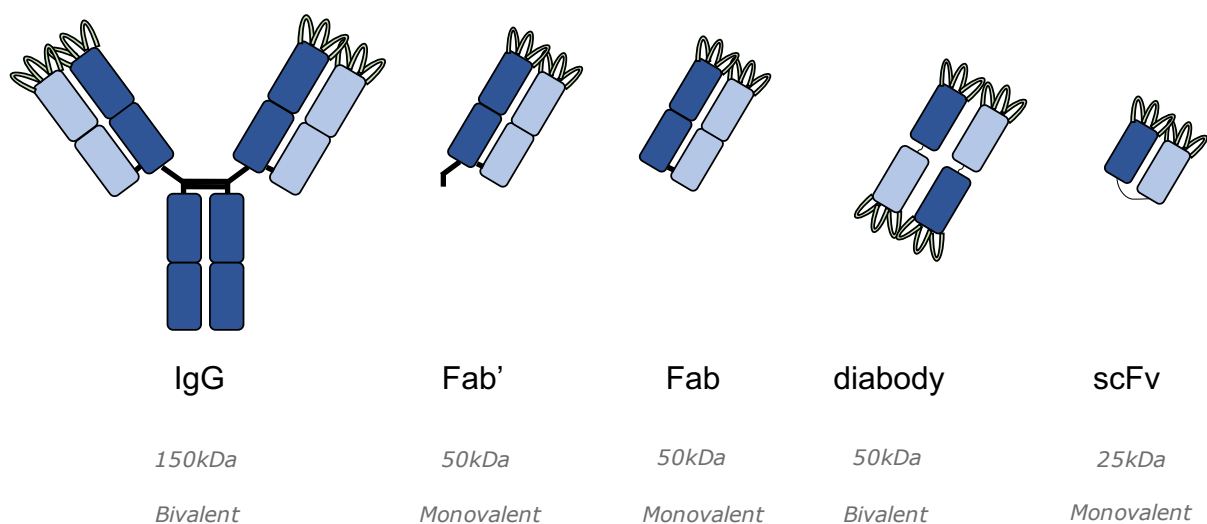


Figure 2.4: Antibodies have a modular structure.

2.2.6 Specificity of antibodies

The rise in antibodies as biotechnology tools has been elicited by the molecules' ability to bind to an unlimited number of antigens which share little or no similarity. Prior to Kohler and Milstein's invention of the hybridoma, polyclonal antibodies (pAb), those that bind to different epitopes of a single antigen, were the mainstay of antibody therapeutics. In comparison, mAbs only recognise one epitope on an antigen. Both mAbs and pAbs have advantages and disadvantages for diagnostics and therapeutics.

pAbs have high affinity and their multi-epitope specificity can lead to an amplification of signal from a target protein making them ideal for diagnostic purposes. They are also tolerant of slight changes (slight denaturation, polymorphism, glycosylation heterogeneity) allowing for the detection of antigens. However, pAbs often have batch-to-batch variability and cross-reactivity issues (Ascoli & Aggeler 2018). On the other hand, mAbs have minimal cross-reactivity due to their highly specific recognition of only one epitope. This also means they are vulnerable to slight changes in the epitope resulting in significantly reduced binding capacity. mAbs provide a source of antibodies that are homogenous in specificity and affinity, making them attractive for therapeutics, where a molecule must be fully characterised prior to use (Thibodeaux et al. 2012). It is worth noting that recombinantly produced mAbs do not suffer from clonal drift or

gene deletion that hybridoma-derived mAbs can experience, while providing the same specificity and reproducibility.

2.3 Immune response to foreign antigens

2.3.1 Innate and adaptive immune response

The innate immune system is the first line of defence for all living organisms. These rely on the body's ability to identify conserved features of antigens that are not present in the host. This is soon followed by the second line of defence, known as the adaptive immune response. The name of the adaptive immune response arises from the need for the response to evolve against the antigen and produce mAbs using class switching and affinity maturation as previously described.

2.3.2 Causes of antibody immunogenicity

Antibody immunogenicity can occur due to both intrinsic and extrinsic factors. Intrinsic factors include but are not limited to glycosylation (Chung et al. 2008), CD4⁺ T-helper cell epitopes (Jawa et al. 2013), deamidation (Verma et al. 2016) and oxidation patterns (Pedras-Vasconcelos 2014). Extrinsic factors such as antibody aggregates (Joubert et al. 2012), process-related impurities, antibody-antibody/drug interactions and the mechanism of action of the antibody can also cause unwanted immunogenicity.

A heightened awareness of the immune responses to antibodies, including humanised antibodies occurred after the PAREXEL (Theralizumab) trial in 2006. In contrast to many other antibodies which act by antagonism, Theralizumab was a humanised anti-CD28 super-agonist. Six first in man trial participants experienced multiple organ dysfunction. This was attributed to severe cytokine release syndrome leading to severe inflammation. This finding was observed at a low level in preclinical primate studies, but overlooked as minimal (Attarwala 2010). This

target-specific cause of immunogenicity highlighted the rapid detrimental effects that can occur from our immune system. Learnings from this incident have changed how first in man trials and clinical trials are conducted and approved.

Foreign antibodies such as rodent antibodies are recognised as foreign resulting in an immune response with the generation of human anti-species antibodies (eg. human anti-mouse antibodies (HAMA)). These antibodies result in the rapid clearance, reduced efficacy (Goldstein & G. 1985), neutralisation and potential risk of inflammatory side effects of rodent therapeutics (Harding et al. 2010). The risk of anti-species antibodies limits the use of non-human mAbs for diagnostics or therapeutics. The discovery that antigen specificity is determined by the CDRs has helped to engineer antibodies with lowered immunogenicity while maintaining beneficial effects. The first engineered improvements were chimeric antibodies, which have human constant regions while maintaining murine variable regions. Increased understanding of the immune response to these chimeric mAbs has become the driving force behind the humanisation of mAbs. Humanised antibodies contain murine CDRs, with the remaining sequence being human. Hwang & Foote (2005) investigated reports of anti-antibody responses over 20 years. The research assigned a ‘marked’ anti-antibody response as one that was reported in greater than 15% of patients. Using this definition, the report found that in 84% of cases there was a ‘marked’ HAMA response, with only a 40% of cases reporting a ‘marked’ human anti-chimeric antibody response. The use of humanised antibodies, showed only 9% of cases with ‘marked’ human anti-human antibody response. Thus, the largest step in reducing immunogenicity comes from the chimerisation of an antibody, however humanising antibodies also lowered these unwanted responses to even lower levels. It is important to note that fully human antibodies may still elicit an immune response. Reasons for this are explored below in the Section “Fully human antibodies”.

2.3.3 Humanisation of antibodies

In the 130 years since Emil von Behring’s seminal work, an increased understanding and appreciation of the severe consequences of the immune response has lead to the humanisation of antibodies.

Despite the initial excitement surrounding muromonab-CD3 (Orthoclone), it turned out to be an intrinsically immunogenic molecule and was withdrawn from the market. Thus, as the importance of antibodies in healthcare has increased, humanisation has become a key step in the process of moving from the pre-clinical to clinical stage of development. The goal of humanisation is to reduce the immunogenicity of the Ig while maintaining the specificity, stability and affinity of the antibody to its antigen (Jones et al. 1986, Verhoeyen et al. 1988, Clavero-Álvarez et al. 2018). The process of humanising antibodies varies with many methods having been described, each with their own advantages and disadvantages. Notwithstanding the presence of computational methods, the process of humanisation is still rooted in trial and error, proving to be a cumbersome step in the development of new imaging and therapeutic antibodies. Until now, there has been no generally applicable technology that can ensure antibodies remain antigenically silent. The subsections below describe some of the more common techniques for humanisation.

CDR grafting onto framework and germline sequences

CDR grafting is the most common method employed, in which the CDRs of non-human antibodies are grafted onto the human frameworks. Generally, the CDRs are grafted onto the human IgG framework sequences with the highest homology of the framework region to the non-human antibody. This method of humanisation of grafting CDRs onto a human framework was patented by Greg Winter in 1991 (Winter 1991). Once the mAb is CDR-grafted, it is likely to be less immunogenic but there is often also a loss of affinity (Pavlinkova et al. 2001).

Foote & Winter (1992) identified specific murine framework residues that were important for the conformation of the CDR loops, and termed these “vernier zone residues”. These residues were generally located in the β -sheet pre and post-CDRs, impacting the CDR packing and hence the affinity of the antibody. The importance of these residues was demonstrated by Makabe et al. (2008) during the humanisation of a murine antibody, termed 528, which had a loss of affinity and resulted in negative effects on both its enthalpy and entropy. Upon employing backmutation of vernier residues, the humanised mAb displayed greatly improved affinity, enthalpy and entropy. The clinically used mAb, Herceptin (Trastuzumab) was successfully humanised using this method and has gone on to be FDA and European Medical Agency (EMA) approved for breast cancer that is HER2-receptor positive (Carter et al. 1992).

A slight variation of this is where CDRs are grafted onto germline human sequences. Germline genes have less intraclonal somatic hypermutation (Pelat et al. 2008) and therefore it has been expected that they will show lower immunogenicity. However, in some cases IgG-derived sequences are more favorable due to different effector functions of isotype subclasses (Thibodeaux et al. 2012).

CDR grafting can be taken one step further where only specificity-determining residues (SDR) are grafted. SDRs are those specific CDR residues that are involved in the interaction with the antigen. In this technique, SDRs are maintained, while other residues are exchanged, similar to the rest of the framework regions (Padlan 1991). The extent of the benefits to which germline humanisation and SDR grafting have above CDR-grafting remains to be addressed clinically.

Other qualities such as the packing angle can be investigated during humanisation. The packing angle impacts the topography of the two chains and is therefore important for maintaining the ABS. Abhinandan et al. (2010) developed a method to calculate the packing angle based on the predicted structure of the sequence inputs. This analysis can be employed for all the methods of humanisation listed below.

Humanisation by resurfacing

Humanisation by resurfacing is based on the HAMA response resulting from the exposed surface residues (Novotny et al. 1986). Thus, the basis of this method involves the replacement of potentially antigenic surface framework residues with the most common human residues at these positions (Padlan 1991). First described by Padlan (1991), antibodies humanised by this method often result in a minimal loss in stability and affinity.

Humanisation by CDR homology

Humanisation by CDR homology is based on the fact that similar canonical CDRs can support the humanised CDR structure while retaining high affinity. In contrast to other methods, the homology of the CDRs is considered while excluding the homology of the framework regions. Antibodies humanised through this method have been found to retain good affinity in comparison to framework-homology based humanisation methods (Hwang & Foote 2005). However, this technique still remains less commonly applied than CDR-grafting.

Guided selection based on chain shuffling

Humanisation using guided selection is based on chain shuffling of V-genes. Mouse VH and VL domains are used to guide the selection of a human VL and VH partner that results in an antibody binding the epitope of the original antibody. This can be performed sequentially or in parallel for both VH and VL domains (Kim et al. 2010). This is one of the only humanisation techniques that can result in a completely human antibody and may generate antibodies with more favourable properties than the original antibody (Jespers et al. 1994). The success of this method was demonstrated with Humira (Adalimumab), which was generated by using the tumor necrosis factor- α (TNF- α) mouse mAb MAK195 as a guide (Osbourn et al. 2005).

Fully human antibodies

Fully human antibodies can be generated through a multitude of techniques. One of the main methods is through the use of transgenic human IgG mice. In these mice the immunoglobulin system has been replaced with a fully human immunoglobulin repertoire generating fully human antibodies from mice (Lonberg et al. 1994). Mice have been the predominant transgenic host species, but the genomes of other models such as rats, rabbits and cows have been modified to express human antibodies. Antibody generation *in vivo* offers several advantages in that somatic hypermutation, quality control checkpoints and *in vivo* sequence diversification is maintained (Lonberg et al. 1994). This often leads to better biophysical properties than *in silico* or phage selected antibodies. Antibodies generated through this method include Illaris (Cankinumab), Repatha (Evolocumab) and Vectibix (Panitumumab).

It is worth noting that an immunogenic response is possible even with fully human antibodies. This may be due to the presence of T-cell epitopes, an overreactive immune system or the presence of non-native post-translational modifications (PTM). The somatic hypermutation (VDJ recombination) can mutate CDRs away from their germline configuration. Serum from non-diseased donors has demonstrated the presence of anti-idiotypic antibody to various autoantibodies (Harding et al. 2010). Anti-idiotypic antibodies are antibodies that have specificity for the variable region of other Ig molecules, including our own.

Fully human antibodies may also be generated from human Ig phage libraries (Breitling et al. 1991, McCafferty et al. 1990, Barbas et al. 1991). Through this method, mAbs can be generated against small or non-immunogenic molecules (Moghaddam et al. 2003) or against slight modifications of the antigen such as phosphorylation (Blokzijl et al. 2016). It would not have been possible to generate these mAbs through classical immunisation.

Since 2015, over 50% of the mAbs approved for clinical use have been fully human (Kaplon & Reichert 2019). While fully human antibodies are the current state of the art, the evidence-based argument for fully human antibodies being less immunogenic than humanised antibodies remains to be substantiated (Harding et al. 2010).

Determining immunogenicity in relation to humanisation

Human string content can quantify the small peptide sequences within the variable domain compared against known human peptide sequences to predict the immunogenicity level of the sequence. This method can enhance the accuracy of engineering during the humanisation process (Lazar et al. 2007). The sequence of antibody heavy and light chains can also be scanned for known T-cell and B-cell epitopes (Griswold & Bailey-Kellogg 2016, Nagata & Pastan 2009). *In silico* techniques tend to be over-predictive but are also important in designing less immunogenic antibodies. The gold standard method for determining immunogenicity is still laboratory-based where the test mAbs are incubated with peripheral blood mononuclear cells from pooled donors (generally 20-50 donors). This allows an analysis of an *in vitro* immune response stimulation of lymphocytes, monocytes and dendritic cells (Groell et al. 2018).

There is an assumption of reduced immunogenicity with greater similarity of the humanised sequence to a true human sequence. This assumption excludes T and B-cell epitopes, which are present in human sequences and can increase the immunogenic potential. Numerous techniques have been developed to estimate the “humanness” of sequences. One of them developed by Abhinandan & Martin (2007) calculates a “z-score” which takes into account the mean sequence identity of a input sequence compared to a dataset of human sequences.

2.4 Expression systems for recombinant antibody fragments

Currently, most therapeutic antibodies are produced in mammalian cell lines to reduce the risk of immunogenicity due to non-human glycosylation patterns. However, recent advances in expression technologies have promised to produce antibodies with PTMs that are “human-like”. In the case of antibody fragments, multiple expression systems can be utilised for their production due to their smaller size and lack of glycosylation. A non-exhaustive overview of some of the available expression systems is explored in the section below.

2.4.1 Prokaryotic expression system

Escherichia coli (*E.coli*) production has historically been the dominant expression system for recombinant proteins. Due to the smaller size of antibody fragments (50kDa and below), they are amenable for production in prokaryotic systems. One of the challenges of production in *E.coli* is the reducing nature of the cytoplasm as it inhibits the formation of intradomain disulphide bridges. This is important in the production of both the Fab and scFv fragments. Thus, antibody fragments have to be refolded under oxidising conditions to generate these bridges (Cabilly et al. 1984). However, aggregation may occur during the refolding process resulting in low yields. Apart from low yields, aggregation is an important characteristic to prevent as it can lead to lower *in vivo* efficacy, variability between batches and can lead to immunogenicity in patients (Cordoba-Rodriguez 2008). Another key limitation of prokaryotic expression of antibody fragments is the need for endotoxin removal, which can reduce final yield and increases steps in the purification process (Mamat et al. 2015, Lin et al. 2005).

More recently, periplasmic expression of antibody fragments has gained popularity as it allows antibody fragments to be expressed and folded in the oxidising environment of gram-negative bacteria (Skerra & Plückthun 1988). However, aggregation may still occur due to high levels of recombinant protein and endogenous proteases. In particular, this affects fragments with low

stability or aggregation propensity including scFvs (Röthlisberger et al. 2005). To combat this and improve protein yields, cytoplasmic disulfide bond formation (CyDisCo) in *E.coli* technology was created where the co-expression of sulfhydryl oxidase and a disulfide bond isomerase allow for natural disulfide bond formation within the cytoplasmic compartment (Hatahet et al. 2010, Gaciarz et al. 2016). Proof of concept fed-batch fermentation demonstrated the production of an scFv with a yield of $139 \text{ mg} \pm 10\text{mg}$ per litre. Additionally, this was carried out in carried out in chemically defined minimal media, in contrast to other cell expression systems such as Origami or SHuffle where the redox system has been perturbed requiring costly media (Gaciarz et al. 2017).

Expression of antibody fragments in gram-positive bacteria such as *Brevibacillus* has been growing, with the benefit of negligible contamination of endotoxins (Anné et al. 2014). These bacteria secrete protein directly into the medium as they lack an outer membrane.

2.4.2 Eukaryotic expression systems

In contrast to bacteria, eukaryotic cells are capable of expressing full-length Igs due to their advanced folding, PTMs and secretion apparatus. However, this is also a limitation as the PTMs can differ greatly from endogenous human antibodies. For example, glycosylation patterns differ substantially in these expression systems which may limit their use in translational work. Murine myeloma cell lines (NS0 and Sp2/0) express antibodies with the galactose- α -1,3—galactose sugar which can result in severe anaphylactic reactions when administered to patients (Chung et al. 2008). Chinese hamster ovary (CHO) cells are widely used due to their safety profile being established and their ability to produce PTMs similar to humans (Durocher & Butler 2009). However, despite this most of the pharmaceutical expression of recombinant proteins is performed in mammalian cells such as CHO and HEK293. This is mainly due the advanced folding and secretion systems making them almost indistinguishable from human secreted proteins.

The productivity of these systems has vastly improved with production yields exceeding 12g

per litre (Frenzel et al. 2013). Serum-free transfection have allowed increased efficiency during transfection as serum proteins may interfere with complex formation (De Los Milagros Bassani Molinas et al. 2014). However, production in mammalian cells is expensive and thus has been largely limited to industrial applications.

Aside from mammalian cells, yeast, insect cells and filamentous fungi are also eukaryotic expression hosts. *Pichia pastoris* (*P. pastoris*) is the major yeast strain used for recombinant antibody production. scFv expression in *P. pastoris* has also been recorded with yields as high as 250mg/L (Eldin et al. 1997). Fabs, requiring two genes for expression, need to be fused and co-transformed making HC to LC ratio optimisation difficult (Humphreys et al. 2002). Newer strains of glyco-engineered yeast are also beginning to be used for commercial antibody production with a more optimal PTM profile (Ye et al. 2011, Zhang et al. 2011). Other expression hosts used include insect cells, filamentous fungi and protozoa.

2.4.3 Stable versus transient transfection

Stable transfection of the chosen expression system is preferable when long-term production of proteins is required. In this approach, the gene integrates with the cellular genome and is passed onto future generations. During early research where multiple candidates need to be generated this is a time-consuming process that is superseded by transient expression. In comparison, transient transfection, the DNA does not integrate and will express the gene for only a short period of time. HEK293 cells are very efficiently transfected with plasmid DNA for transient transfection (Ooi et al. 2016) and hence are optimal for early research and discovery.

2.5 Atherosclerosis

Atherosclerosis is a chronic inflammatory disease of the arteries triggered by the retention of Apolipoprotein B (ApoB) within the subendothelium (Libby 2002). The progression of the disease is slow, usually taking several decades to produce advanced lesions. It is characterised by

various cellular changes at different stages of lesion development. The vast majority of lesions remain asymptomatic. However, those lesions can cause thrombosis, impair and result in a MI, stroke or sudden death. The global impact of atherosclerosis is manifested with cardiovascular disease being the most prevalent disease worldwide (WHO 2017).

There are two main components of the immunology of atherosclerosis, the innate and adaptive immune response. The innate being memory-cell independent, and the adaptive being memory-cell dependent (Hansson et al. 2002). The pathobiology of atherosclerosis is explored further in the section below.

2.5.1 Pathobiology of Atherosclerosis

Atherogenic stimuli such as abnormal shear stress and tobacco smoke can induce endothelial dysfunction through a decrease in nitric oxide (NO) and increase in reactive oxygen species (ROS). This shift from a protective state plays an important role in the initiation of atherosclerosis as the ensuing endothelial activation results in increased vessel permeability, upregulation of cellular adhesion molecules and the secretion of pro-inflammatory cytokines. The increased endothelial permeability allows LDL deposition within the subintimal space (Figure 2.5). Here, LDL can be oxidised as it is shielded from the anti-oxidant environment of the circulation and exposed to a pro-inflammatory environment. This modification renders LDL antigenic allowing it to be recognised by pattern-recognition receptors on macrophages (Brown et al. 1979).

In the early stages of atherosclerosis, the inflammatory environment recruits leukocytes to the area of vascular injury, causing monocytes to enter the intima and differentiate into macrophages (Babaev et al. 1993). These plaque macrophages further produce inflammatory mediators recruiting more immune cells to the developing plaque. The uptake of oxLDL is mediated by scavenger receptors on the macrophages. Scavenger receptors lack a negative feedback loop that controls the physiological LDLR, allowing for the unregulated uptake of LDL (Brown et al. 1979). The hyperlipidemia limits the ability of macrophages to clear the lipid particles and they become lipid-overloaded, in turn receiving the name “foam cells” due to the

accumulation of cholesteryl ester droplets within the cytosol. These foam cells contribute to the highly inflamed environment, activating inflammation pathways and producing atherogenic cytokines and matrix metalloproteinases (Newby 2008).

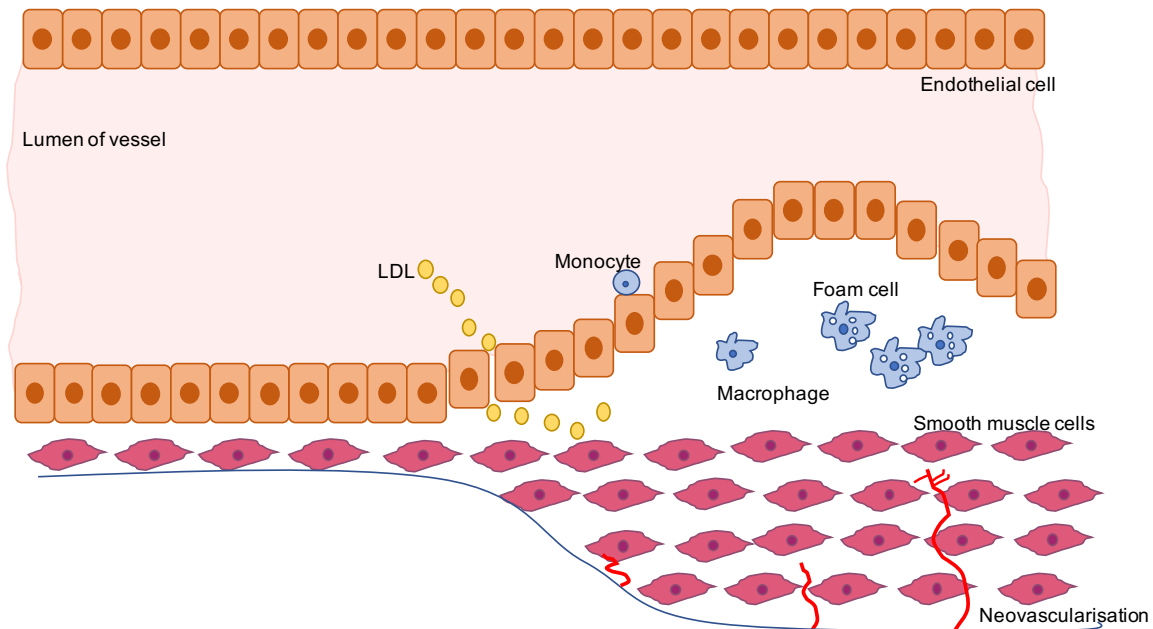


Figure 2.5: Pathobiology of atherosclerosis. In regions of non-laminar blood flow, an upregulation of inflammatory mediators is triggered resulting in endothelial dysfunction. This dysfunction allows for the infiltration of LDL into the sub-endothelial space. Here, LDL becomes modified, rendering it antigenic. This antigenic form is taken up by macrophages, creating foam cells and as they apoptose they aid in the formation of the necrotic core. Further progression of the disease can lead to narrowing of the vessel and/or thrombosis.

As the plaque progresses, foam cells undergo apoptosis resulting in the build-up of necrotic debris forming the “necrotic core”. This formation triggers further release of inflammatory cytokines including $\text{TNF-}\alpha$ and interleukin-6 (IL-6). This continues the progression of atherosclerosis by inducing the growth of vascular smooth muscle cells (VSMC) leading to the narrowing of the lumen (Libby 2002, Sherer & Shoenfeld 2006). Neoangiogenic vessels that develop allow the plaque to grow by providing further nutrients and immune cells. However, these new vessels are fragile and bleed resulting in intraplaque hemorrhages, thereby aggravating the inflammatory state which may lead to plaque rupture (Kolodgie et al. 2003).

Inflammation plays a key role in the instability of the plaque. The production of proteinases degrades the extracellular matrix of the plaque fibrous cap (Galis & Khatri 2002, Aikawa 2015),

with matrix metalloproteinases (MMP) and cathepsins making up the major classes of expression (Galis et al. 1994, Herman et al. 2001). The thinning of the cap can result in the rupture of the plaque, exposing thrombogenic material to the blood and often resulting in clinical events. It is worth noting that plaques generally enlarge outward at first and thereby preserve the lumen. This is known as positive remodelling. As the lesion develops, the remodelling becomes “negative” and begins to obstruct the flow of blood.

2.5.2 The role of LDL and its oxidation in atherosclerosis

Lipoproteins are composed of both proteins and lipids and are responsible for ensuring a supply of cholesterol to cells and tissues. There are five major classes of lipoproteins: chylomicrons, very low-density lipoproteins (VLDL), LDL, intermediate density lipoproteins (IDL) and high density lipoprotein (HDL). LDL is of key interest to researchers as it is the primary form of transport for cholesterol and has been shown to have a principal role in the pathophysiology of atherosclerosis (Prassl & Laggner 2009, Libby 2002). LDLs form a heterogeneous group of particles, which range in surface charge, chemical composition and size, between 18-25nm with an average size of 22nm (Chapman et al. 1999, Mcnamara et al. 1996). Despite their heterogeneity, LDL molecules all contain three key components: a lipid core containing cholesteryl esters and triglycerides, the protein ApoB and a phospholipid monolayer. LDLs are formed as a consequence of VLDL transporting triglycerides to muscle and adipose tissue. VLDL is hydrolysed by lipoprotein lipase (LPL), which removes triglycerides for energy production or storage. This in turn affects the molecule’s composition and generates a molecule known as IDL, which can either be internalised by the liver or become Low density lipoprotein (LDL) via the loss of Apolipoprotein E (ApoE). LDL can then be endocytosed by cells through the LDLR, which recognises the ApoB moiety (Zhang & Ho 2016), and is used by cells for their various functions such as hormone production and maintenance of the cellular membrane (Sherer & Shoenfeld 2006, Prassl & Laggner 2009). In a pathological state, the cellular uptake of LDL is mediated by scavenger receptors (Brown et al. 1979).

Upon macrophage uptake, cholesterol is esterified and stored in lipid droplets, which can later be de-esterified into free cholesterol and be transported to HDL for reverse cholesterol transport. Intracellular cholesterol crystallisation and release of these crystals into the intracellular space may occur when the generation of free cholesterol is greater than the capacity of HDL removal. These crystals are capable of further initiating inflammation through the activation of the NLR Family Pyrin Domain Containing 3 (NLRP3) inflammasome and expression of IL-1 β which may result in plaque destabilisation.

Due to the heterogeneous population of LDL particles, there are disparities in regards to receptor binding affinity and susceptibility to modifications. For example, smaller and denser LDL particles have demonstrated increased unspecific binding and lower affinity to the LDLR (Prassl & Laggner 2009). It is these small LDL molecules that have been recognised as a risk factor for atherosclerosis, as they are more susceptible to modifications and thus more atherogenic than their larger counterparts (Packard 2006). This may be due to their smaller size being more capable of entering the subendothelium than larger counterparts. Transgenic animals that lack the LDLR gene (LDLR^{-/-}) or have it disrupted demonstrate accelerated disease (Oppi et al. 2019). This is the experimental model used in this thesis.

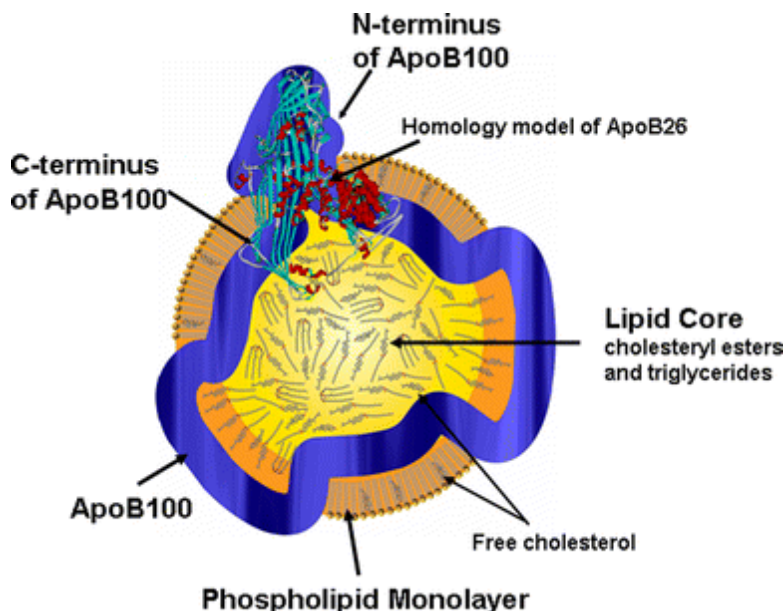


Figure 2.6: Schematic diagram of a LDL particle. A LDL particle consists of a lipid core containing cholesteryl esters and triglycerides. A single molecule of Apolipoprotein B-100 wraps around this lipid core along with a single layer of free unesterified cholesterol. Image from Prassl & Laggner (2009) with permission.

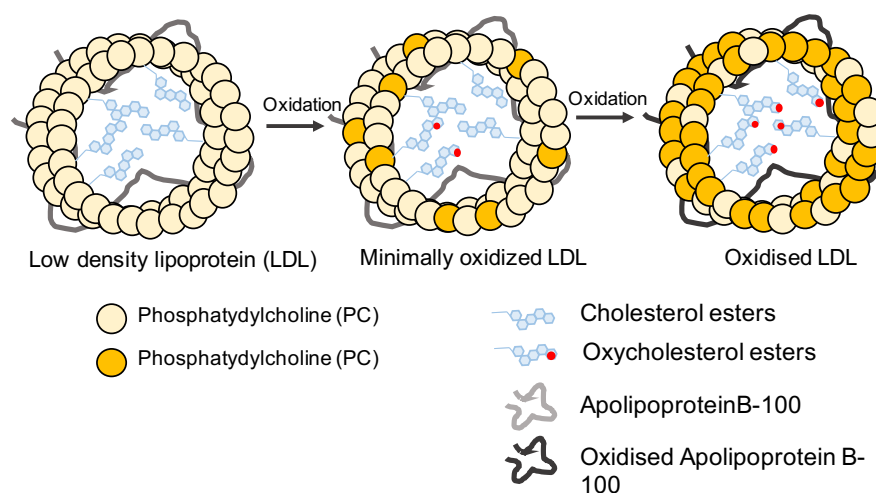


Figure 2.7: Spectrum of oxidative states of LDL particle. Minimally oxidised LDL involves the modifications of the cholesterol esters and lipid molecules to produce lipid oxidation products. In heavily oxidised LDL these lipid products react with Apolipoprotein B-100 resulting in these adducts modifying the protein structure itself. Adapted from Zmysłowski & Szterk (2017) with permission.

LDL oxidation is understood to occur in two steps as shown in Figure 2.7. The first being minimally-modified LDL, in which there is a lack or only slight modification to apoB-100, and the modified LDL retains its affinity to the LDLR (Zmysłowski & Szterk 2017). However, this form of LDL may induce inflammatory changes and activate anti-apoptotic signalling thus promoting further oxidation. With further oxidation of both the lipid and protein moieties leading to modifications of the secondary structure of the protein, the LDL particle is no longer recognised by the LDLR (Goldstein et al. 1979). Additionally, this heavily oxidised LDL can ligate scavenger receptors such as SR-A, CD36, SR-PSOX and LOX-1 (Yamada et al. 1998, Steinberg 1997) with high affinity (Hansson et al. 2002).

2.5.3 Importance of oxLDL in atherosclerosis

Non-laminar blood flow and vascular insult results in endothelial dysfunction and the subsequent upregulation of inflammatory mediators (Davis et al. 2001). In these regions, lipoproteins can pass through the endothelial barrier and enter the intima from circulation. This lipoprotein deposition in the arterial wall drives the initiation and progression of the disease (Newby 2016). The early lesions, known as fatty streaks, are reversible and clinically

insignificant. As the disease progresses, the interaction between ApoB and intimal extracellular proteoglycans prevents the movement of lipoproteins resulting in their buildup. The retained lipoproteins are subject to modifications by oxidising agents, lipases and proteases generating endogenous antigenic neo-epitopes (Nakashima et al. 2008). This modification of LDL forms a pathological entity which is central to the pathogenesis of atherosclerosis and can further induce its aggregation (Goldstein et al. 1979). The disease is self-perpetuating.

Administration of antibodies blocking the uptake of oxLDL by macrophages resulted in up to 57% less atherosclerosis than in controls (Que et al. 2018). Furthermore, antibodies which block the intimal retention of LDL decreased LDL modification and atherosclerosis (Newby 2016). Even in cases of advanced atherosclerosis, plaque regression is accompanied by lower rates of atherosclerotic cardiovascular disease events (Tang et al. 2016, Rosenson et al. 2018).

It is worth noting that oxLDL is not a single molecule, rather a spectrum of differentially modified LDL molecules. These include MDA-LDL, enzymatically-modified LDL, minimally modified-LDL and electronegative LDL (Levitan et al. 2010). In the next section, MDA-LDL will be further discussed as the main oxLDL entity of this thesis. Its role in atherosclerosis (Virmani et al. 2006, Matsuo et al. 2012, Tajika et al. 2012), inflammation (Bentzon et al. 2014) and apoptosis (Virella et al. 2018) among others is extensively published.

2.5.4 Vulnerable and stable plaques

Clinically, not all atherosclerotic lesions are considered equal. Stable plaques have a dense, collagenous fibrous cap with a small lipid core and minimal inflammation. On the other hand, vulnerable plaques have a large lipid core (greater than 40% of plaque's volume), with a thin fibrous cap (less than 65-55 μm) and increased levels of inflammation (Virmani et al. 2006). It is these atherosclerotic lesions that concern clinicians, as they can rupture and result in a stroke or MI. Vulnerable plaques are also known as thin-capped fibroatheromas (TCFA). Stable plaques that impede blood flow are also of concern to clinicians, but as they are generally symptomatic, they are more easily diagnosed. They are characterised by a small lipid core,

thick fibrous cap and lower levels of inflammation (Virmani et al. 2006).

Malondialdehyde LDL

Lipoproteins are susceptible to oxidative damage by free radicals resulting in changes to biological properties (Goldstein et al. 1979). In the process of lipid peroxidation, highly reactive aldehyde products including Malondialdehyde (MDA) are generated. These highly electrophilic lipid oxidation products are reactive with nucleophilic groups allowing them to attach with biological molecules such as LDL (Afonso & Spickett 2019). This forms an antigenic epitope. As early as the 1980s, MDA-LDL was demonstrated to be taken up by macrophages as these particles shifted from being recognised by the native LDLR to the scavenger receptor (Goldstein et al. 1979). This process was subsequently demonstrated in both human and animal models (Haberland et al. 1988) with the presence and levels of MDA-LDL reflecting the severity of coronary artery disease (Amaki et al. 2004, Viigimaa et al. 2010, Tajika et al. 2012, Matsuo et al. 2012).

Evidence is growing demonstrates that blood MDA-LDL levels correspond with plaque vulnerability (Matsuo et al. 2012, Tajika et al. 2012). However, the presence of a vulnerable plaque as described previously may not always lead to clinical events (Bentzon et al. 2014). Nonetheless, several studies have demonstrated that plaque characterisation and identification of vulnerable plaques may predict future events (Kim et al. 2009, Wykrzykowska et al. 2012). Molecular imaging techniques in combination with anatomic techniques may aid in this venture by improving plaque characterisation, leading to more clinically robust predictions for future clinical events and hence treatment guided decisions.

2.5.5 Macrophage handling of modified LDL

Monocytes attracted by pro-inflammatory signals migrate to sites of endothelial dysfunction and infiltrate the intima (Virmani et al. 2006, Larmann et al. 2013). Macrophages express

scavenger receptors such as class A (type I, II, MACRO, SRCL), class B (CD36, SR-BI), class D (CD68), class E (lecitin-like OxLDL receptor-1 (LOX-1)), class F (SREC), class G (SP-PSOX) and the Fc receptor family (PrabhuDas et al. 2014). The recognition of oxLDL by these receptors, and the lack of its recognition by the physiological LDLR causes the accumulation of cholesterol esters in the cytoplasm of macrophages and the eventual formation of foam cells (Palinski et al. 1989). Macrophages produce cytokines such as IL-1 and TNF- α further driving inflammation and oxidative modifications of lipoproteins generating a vicious negative cycle. Reduced atherosclerosis is hyperlipidemic mice lacking SR-A and CD36 receptors implicates these pathogen-associated molecular pattern recognition molecules in the disease (Afek et al. 2000, Zhou et al. 2006).

Pro-protein convertase subtilisin/kexin type 9 (PCSK9), an enzyme which plays a role in the degradation of LDL receptors has been demonstrated to have elevated levels in human and mouse atherosclerotic plaques (Stawowy 2015, Gustafsen et al. 2014). Human macrophages demonstrated an upregulation of PCSK9 in the presence of oxLDL (Tang et al. 2012). Evolocumab, a PCSK9 inhibitor is showing great promise for statin-intolerant patients or for synergistic use with statins to further lower LDL levels (Reiss et al. 2018).

Importantly, scavenger receptor mediated uptake may not be possible when LDL is adherent to the matrix. Here, activated macrophages are thought to promote the breakdown of the extracellular matrix through the release of lipolytic and proteolytic enzymes including cathepsins and lysosomal acid enzymes (Buton et al. 1999).

ATP-binding cassette (ABC) transporters ABCA1 and ABCG1 and SR-BI contribute to the reverse transport of cholesterol from macrophages. HDL or ApoA1 capture this released cholesterol (Chistiakov et al. 2016). In ApoE deficient (ApoE^{-/-}) mice, deletion of SR-BI resulted in advanced atherosclerosis and overexpression of this receptor delayed formation of plaques (Zhang et al. 2003). This is replicated in LDLR^{-/-} where overexpression of SR-BI protected against advanced atherosclerosis, albeit increasing early atherosclerosis (Van Eck et al. 2004). This is likely due to its bidirectional role where SR-BI behaves similarly to SR-A1 to deplete

intracellular cholesterol levels.

2.6 Overview of current preclinical and clinical strategies for imaging atherosclerosis

The insidious nature of atherosclerosis makes it difficult to detect until a clinical event occurs. Imaging has retained its central role in detection, treatment and prevention of atherosclerotic disease. Historically, angiography has been the gold-standard for diagnosing obstructive vascular disease as anatomic imaging is routine for the assessment of blood vessel structure and luminal stenosis. Anatomic techniques also provide insights about plaque composition but they are unable to define critical biologic components of plaque physiology that predispose them to rupture (Guo et al. 2021, Jones et al. 2021). Conversely, molecular imaging permits the visualisation and quantification of key determinants of atheroprogession. Many of the techniques employed, such as computed tomography (CT), magnetic resonance imaging (MRI), intravascular ultrasound (IVUS), position emission tomography (PET) and single photon emission computed tomography (SPECT) can combine both structural and functional imaging to give advanced diagnostics allowing earlier diagnosis and better disease progression monitoring. Imaging of coronary arteries poses challenges due to their small size requiring high spatial resolution to determine local plaque biology, as well as high temporal resolution to avoid blurring due to movement from the heart beat and breathing. Large vessels are less susceptible to these motion artefacts.

2.6.1 Anatomic imaging

X-Ray CT is a non-invasive method using high resolution x-ray imaging in combination with intravenously administered iodinated contrast agents to visualise cardiac and vascular structures. It is quick and non-invasive, however spatial resolution is low, therefore invasive angiography has greater sensitivity for recognising obstructive stenosis and is often preferred. However, angiography does not provide information about blood vessel wall structure or size of

plaques below the endothelial surface and thus would not detect positive remodelling in early atherosclerosis (Gorenoi et al. 2012).

MRI gives non-invasive imaging of cardiovascular structures, with high spatial and temporal resolution. It lends well to imaging of large arteries and veins, but clinical use on coronary arteries remains limited due to their small size. Recent advances with 3 Tesla MRI scanners have renewed research into clinical diagnostic imaging of coronary arteries (Swirski et al. 2007). Within research, invasive MRI coil detectors have allowed the characterisation of TCFAs with specificity and sensitivity (Larose et al. 2005). Gerretsen et al. (2010) demonstrated that MRI was sensitive to early atherosclerotic vessel luminal narrowing in asymptomatic patients. Moreover, Noguchi et al. (2014), demonstrated high intensity non-contrast T1 weighted coronary MRI to be a significant predictive indicator of future outcomes. Furthermore, novel motion correction and reconstruction techniques have revived interest in coronary magnetic resonance angiography (Bustin et al. 2020). This would allow non-invasive monitoring of early disease and more selective therapeutics in high-risk patients.

IVUS is an invasive technique that has high spatial resolution and depth of penetration and can provide in-depth vascular information including precise vessel dimensions (Nishimura et al. 1990). An extension of IVUS is IVUS-virtual histology (IVUS-VH) which generates a color-coded image of plaque composition onto the grey-scale IVUS image improving characterisation of lipid content and the necrotic core (Claessen et al. 2012).

Optical coherence tomography (OCT) is an invasive intravascular near infrared-red (NIR) light imaging technique that generates a cross-sectional image of the vessel in greater spatial resolution than IVUS. However, NIR light does not penetrate as deeply as sound and hence this technique has a low depth of penetration (Kubo et al. 2011). Advances in catheter speed have allowed OCT imaging to be a high-resolution, convenient technique used widely clinically, despite the NIR light scattering by red blood cells. Studies have established that OCT is effective for identifying and quantifying TCFAs (Kubo et al. 2011, Bouki et al. 2012).

IVUS and OCT provide methods to image plaque anatomy. Studies have demonstrated that

OCT can identify and quantify TCFAs effectively (Kubo et al. 2011). IVUS and OCT currently lack the ability to determine important biological targets which promote vulnerability. Recently, optoacoustic, or photoacoustic imaging, has demonstrated an increased contrast-to-noise ratio due to advances in laser technology. This has made optical high resolution visualisation within deep tissue possible. Xie et al. (2020) demonstrated significantly higher levels of anti-CD36 nanoparticle in atherosclerotic mouse carotid arteries as compared to normal and non-targeted nanoparticles using non-invasive photoacoustic imaging. In a proof of concept study, a human carotid artery inside a neck phantom demonstrated clear photoacoustic signal at 1200nm due to the peak lipid absorption wavelength of 1210nm (Zafar et al. 2018, P et al. 2014, Caplan et al. 2006). Thus, these studies demonstrate the potential of combining different imaging modalities to improve patient outcomes.

2.6.2 Molecular Imaging

Molecular imaging techniques, also known as “functional imaging” allows the visualisation of biological processes at the molecular levels. The advantage of molecular imaging over anatomical imaging is the ability to characterise the biological determinants of disease, rather than only anatomical features. This may lead to more personalised treatment regimens dependent on the individual pathophysiological profile of a patient.

With its roots anchored in cancer, the field of molecular imaging is rapidly developing and growing in other diseases. It has greatly expanded the understanding of disease pathobiology where it has been implemented. Recent technological advances in nuclear medicine, MRI, near infrared fluorescence (NIRF) and ultrasound have fueled its growing use. Progress in the chemical modifications of proteins and antibodies have allowed the development of novel targeted probes detecting specific cell types or biological processes (Aikawa 2015).

Key frameworks for the successful molecular imaging development to occur include:

- 1) A target molecule that is well understood,
- 2) Imaging agent with high specificity for target molecule,
- 3) High expression of target molecule seen in diseased areas,
- 4) Low background signal from blood pool,
- 5) Imaging device or method,
- 6) A model in which to test these components.

Table 2.2: Imaging modalities stratified by nature of invasiveness at current stage of use. Adapted with permission from Aikawa (2015).

	Modality	Technology	Spatial Resolution	Depth of Penetration	Imaging moieties
Non-invasive	CT	X-ray	0.5mm	No limit	Iodinated molecules
	MRI	Magnetic	1mm	No limit	Gd chelates, SPIO, USPIO
	PET	Nuclear	~2mm	No limit	18F, 64Cu, 11C, 99mTc, 123/124/15/131I, 111In
	SPECT				
	Bioluminescence	Optical	3-5mm	1-2mm	Flourescent genes
	Flourescence	Optical	2-3mm	1mm	Conjugated fluorphores
	FMT	Optical	1-3mm	2cm	Conjugated fluorphores
Invasive	Angiography	X-ray	~0.5-1mm	No limit	Iodinated molecules
	OCT	Optical	~10µm	1-3mm	None
	Angioscopy	Optical	10-50µm	Surface imaging	None
	IVUS/IVUS-VH	Ultrasound	~150mm	4-10mm	None

2.6.3 Molecular Imaging of Atherosclerosis

Molecular phenotyping of a plaque can be performed with an array of imaging modalities. Molecular imaging methods utilise specific imaging probes which are linked to detectable imaging constituents.

Non-optical imaging modalities

Macrophages are the principal immune cells in plaques, thus garnering much attention for imaging. Ultrasmall superparamagnetic iron oxide (USPIO) and superparamagnetic iron oxide

(SPIO) have been used for imaging cell activation through the use of anti-VCAM-1 peptide or antibody attachments (Burtea et al. 2012, Rucher et al. 2019). These nanoparticles are taken up by phagocytic cells, preferentially accumulating in cells such as macrophages. The use of this was demonstrated in the Atorvastatin Therapy: Effects on Reduction of Macrophage Activity (ATHEROMA) study which demonstrated a reduced plaque USPIO uptake being reflective of decreased macrophage-mediated plaque inflammation in humans (Tang et al. 2009). A proof-of-concept study using CT to image inflammation demonstrated an increase in macrophage density using both morphologic and functional information (Hyafil et al. 2007). However, the radiation dose used was high and may not be acceptable for routine clinical use.

Studies have shown that plaques have increased metabolic activity as compared to healthy vascular tissue (Hanssen et al. 2014). Thus, fluorodeoxyglucose (FDG) PET has been employed as it readily accumulates in metabolically active cells (Folco et al. 2011). Promising pre-clinical rabbit studies have demonstrated that atherosclerotic rabbit aortas have increased ^{18}F -FDG PET signal compared to control animals (Tawakol et al. 2005). However, biological issues such as the contribution of various cell types to FDG uptake remain to be resolved.

Optical Imaging modalities

Unfortunately, the majority of the imaging techniques used within the biomedical field today rely on deep-penetrating radiation, which can limit repeated imaging. Furthermore, they can have limited spatio-temporal resolution and may lack probes for specific biological targeting. Optical imaging utilises the properties of light, such as absorption, emission, scattering, and fluorescence, as a source for generating contrast images.

The progression of fluorescence imaging to clinics has been limited by its low photon penetration depth. However, the advent of new technologies has allowed imaging further into the NIR than was previously possible. An example of this is imaging in the near-infrared range (700-1,700nm) which provides the advantages of reduced blood attenuation and reduced autofluorescence, compared to imaging at other wavelengths as shown in Figure 2.8. It allows

high-fidelity imaging with greater tissue penetration than the visible or ultraviolet range. Until recently, the NIR was defined as between 700-900nm (NIR-I), but this has been increased to 1700nm (NIR-II) due to advancements in the field.

Fluorophores such as indocyanine green (ICG; emission 800nm) are able to perform structural and functional imaging in the gastrointestinal tract, blood and lymphatic vessels and ophthalmic angiography (Hong et al. 2017). Directly related to this thesis, ICG has also been shown to localise in atherosclerotic plaques. However, the authors were unable to definitively elucidate the biological targeting of ICG, thereby reducing its potential for *in vivo* human use (Verjans et al. 2016). Fluorescence imaging of the vasculature has been aided by the development of intravascular NIRF catheters. Uniquely, intravascular NIRF was able to precisely identify mild lesions in rabbit aorta which was not detectable with traditional angiography (Jaffer et al. 2011).

In Vivo Imaging System (IVIS) is a commonly used optical molecular imaging modality for experimental models. However, its effectiveness *in vivo* at recognising signalling events remains to be established, with research focused on demonstrating correspondence between *ex vivo* and histological samples (Seifert et al. 2018). The quantification of an *in vivo* tracer is also not possible using IVIS. An emerging technique is fluorescence molecular tomography (FMT) which can be a great alternative to standard molecular imaging techniques as it can acquire high sensitivity detection with low instrumentation expense. Ntzichristos (2006) demonstrated that fluorescent signals could propagate through human tissue, thereby setting the stage for this non-invasive technique to be used in clinics. Its translation to clinical use has been aided by the development of probes in the near infrared range (650-900nm). These are characterised by low attenuation in tissues allowing light to propagate several centimetres and with a low radiation hazard (Delpy & Cope 1997). In contrast to planar imaging, which cannot resolve depth and take into account non-linear signal resulting from propagation, FMT is able to obtain data and project this over 360 degrees.

FMT technology easily integrates with current laboratory technologies. For example, it allows

confocal microscopy validation of tissue after *in vivo* imaging. The different wavelengths and available probes allow the interrogation of multiple markers simultaneously. Proper quantification has been hindered by light propagation through tissue being non-linear, but in FMT this is accounted for by the large number of independent data samples and the number of detectors used (Stucker et al. 2011). Thus, FMT can provide molecular and functional information similar to PET, but similarly suffers from poor spatial resolution similarly. Another limitation of FMT is the penetration of depth of light into humans and larger experimental models. This has been largely overcome by a new-wave of catheters for intravascular imaging (Jaffer et al. 2011).

Studies thus far performed with FMT for atherosclerosis have been predominantly performed in ApoE^{-/-} mice. They have focused on the characterisation of cathepsins and MMPs (Deguchi et al. 2006) and the effects of atorvastatin therapy (Larmann et al. 2013). Thus, the study of atherosclerosis in LDLR^{-/-} mice investigating oxLDL is novel use case.

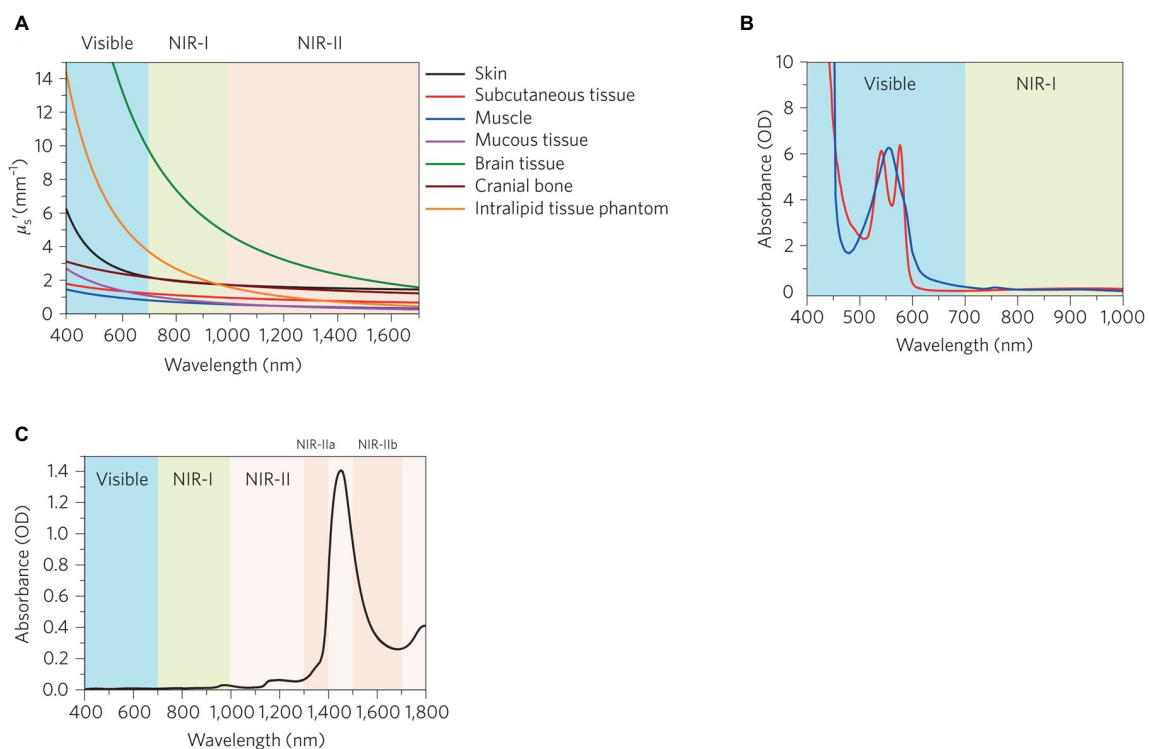


Figure 2.8: NIRF-based imaging has low autofluorescence. A) Reduced scattering coefficients in various biological tissues covering the visible and NIR windows. The longer the wavelength the more reduced the scattering is. B) Absorption spectra of oxyhaemoglobin (red) and deoxyhaemoglobin (blue) show reduced absorption near zero in human blood through a 1 mm long path length. C) Absorption spectra of water is low in the NIR-1 and increases only in NIR-2 range. 1mm long path length. Panel adapted from Hong et al. (2017)

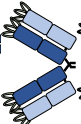
2.6.4 Antibody or antibody fragments for molecular imaging

Antibodies are superior targeting molecules and harnessing this specificity is the goal for both therapeutics and molecular imaging. However, the large size of IgG and interaction with the neonatal Fc receptor (FcRn) (Li et al. 2017, Kontermann 2016), results in serum half-lives up to 2-3 weeks. This makes whole IgG impracticable for clinical imaging purposes due to signal to noise ratio issues (Xenaki et al. 2017, Khamis et al. 2016).

The advent of antibody engineering has given rise to a host of antibody formats with different properties as shown in Table 2.3. The ability to modify features such as molecular size, binding affinity, pharmacokinetics and valency makes antibody fragments well-suited for a variety of clinical applications. Their small size and lack of an Fc region make antibody fragments ideal for imaging as they give high contrast in a short time frame due to biological inertness and size. Improvements for imaging due to the increased vessel permeability may also be possible but this may also lead to non-specific accumulation. Fragments also allow imaging with radionuclides due to short half-lives, thereby, reducing radiation exposure (Richards et al. 2016).

Antibody fragments greater than the renal cut-off of 60kDa benefit from enhanced permeability and retention as they bypass renal filtration. This increases their likelihood of extravasation (Kim et al. 2014). However, data have demonstrated that there is decreased diffusivity as the size of Ig molecules increase (Galgoczy et al. 2014). Thus a fine balance must be struck with these larger antibody fragments.

Table 2.3: Properties of antibody formats. Different antibody formats and their typical properties in humans. Table adapted with permission from Freise & Wu (2015).

Format	Composition	Valency	Approx MW (kDa)	Typical Serum $t_{1/2}$	Clearance Route	Reference
	Intact IgG (VH + CH1-3 + VL + CL) ₂	Bivalent	150-160	1-3 weeks	Hepatic	Ogasawara et al. (2013)
	F(ab') ₂ (VH + CH1 + VL + CL) ₂	Bivalent	110	8-10h	Hepatic	Lütje et al. (2015)
	Minibody (scFv + CH3) ₂	Bivalent	75	5-10h	Hepatic	Tavaré et al. (2014)
	Fab VH + CH1 + VL + CL	Monovalent	50-55	12-20h	Renal	Chakravarty et al. (2014)
	Diabody (scFv) ₂	Bivalent	50	3-5h	Renal	Tavaré et al. (2014)
	scFv VH + VL	Monovalent	28	2-4h	Renal	Kim et al. (2014)

2.6.5 Use of antibodies for imaging atherosclerosis

Antibody-based imaging of atherosclerosis has predominantly targeted oxLDL as it plays a critical role in the pathogenesis of atherosclerosis. One of the first antibodies used for imaging of atherosclerosis was IK17, a monoclonal Fab which binds MDA-LDL and copper-oxidised LDL (cu-oxLDL). It was shown to target the necrotic core of atherosclerotic lesion in human and rabbit arteries and possibly block the uptake of oxLDL by macrophages (Shaw et al. 2001). It was also found to localise to advanced atherosclerotic lesions. The group that has carried this work forward has generated Fab and scFv versions of IK17. However, rather than concentrating on imaging, they have investigated the therapeutic role that the fragments may possess (Tsimikas et al. 2011). Another anti-oxLDL antibody was conjugated to a NIR797 dye and used for imaging of atherosclerosis in ApoE^{-/-} mice. This study was qualitative in nature, but it demonstrated localisation of NIRF in common lesion sites in the aortas of mice (Lu et al. 2014).

Previous uses of antibodies in atherosclerosis imaging include a preclinical rabbit atherosclerotic model, where a ⁹⁹Tc-labelled anti-LOX-1 antibody quantified plaques by PET. Although this was not correlated with histological plaque grading it did correlate with post-mortem LOX-1 plaque expression (Ishino et al. 2008). Anti-oxLDL antibodies have allowed imaging of plaque progression and drug-induced plaque regression (Lu et al. 2014), thus demonstrating the potential of monitoring and targeting plaques with antibody-drug conjugates with this method.

Antibodies with other specificities have also been utilised for imaging atherosclerosis. These include an anti-VCAM 1 for MRI imaging (McAteer et al. 2007), anti-fibronectin mAb using radioiodine or NIR fluorophores (Matter et al. 2004) and anti-fibrin mAb for PET imaging (Greco et al. 1993). Bertrand et al. (2019) demonstrated the feasibility of using anti-ICAM 1 single domain antibodies for IVUS-NIRF imaging. This showed significant promise with only two rabbits showing some non-specific binding in negative controls.

2.7 Work leading up to this project

In this chapter, I have presented case for the use of antibodies to advance the field of diagnostics and therapeutics of atherosclerosis. In addition, I have presented the field of atherosclerosis imaging as one with a current gap in imaging and targeted therapeutics. Taken together, this details the motivation of this project, with further details into the previous work of our research group detailed in this section.

Antibodies have been previously shown to target oxidation specific epitopes (OSEs), including phosphocholine and LDL. These have been used as biomarkers of OSEs and have been used for imaging in animal models. Our group has previously generated IgG monoclonal autoantibodies from a non-immunised one year-old LDLR^{-/-} mouse. Hybridoma cells were generated from its spleen and screened for reactivity against different forms of LDL including cu-oxLDL. LO1, an IgG3k mAb was found to react against cu-oxLDL and MDA-LDL, but not with unoxidised LDL (Chang et al. 2012). Immunohistochemistry and *in vivo* studies in a LDLR^{-/-} mouse model demonstrated that LO1 penetrated the endothelium and specifically labelled lesions and macrophage-laden areas of the arterial vessels (Khamis et al. 2016). As presented in the previous sections, MDA-LDL has been identified in ruptured plaques in both human and animal models (Virmani et al. 2006, Amaki et al. 2004). Given this, it was hypothesised that LO1 may be used as a probe for identifying plaques that may be prone to rupture. While *in vivo* studies demonstrated that LO1 localised to the aortic arch region, a common site of atherosclerosis, it also demonstrated a low signal to noise ratio due to high background arising from its long half-life. Additionally, the murine nature of LO1 would likely lead to a HAMA response in patients as described in Section 2.3.2. These undesirable characteristics are a major challenge for further clinical translation and regulatory approval. Therefore, this project aimed to identify antibody formats of LO1 that may be used for diagnostic imaging of atherosclerosis in the first instance. This involved identifying antibody formats which would have a desired activity profile and reduced antibody immunogenicity. To achieve this aim, the following were hypotheses were investigated:

- A humanised LO1 will have reduced immunogenicity in order to begin its journey for regulatory approval.
- Due to its smaller size, an antibody fragment will lead to better tissue penetration and signal-to-noise ratio *in vivo*.

As previously discussed, humanisation of mAbs is a route to reduce the immunogenicity of an identified mAb. The antibody fragments pursued in this thesis include the Fab, scFv and diabody format. The Fab and diabody format are both of similar molecular weight at approximately 50kDa. However, the Fab is monovalent, and the diabody is bivalent with potentially greater avidity. The scFv was also chosen as a promising format due to its significantly smaller size at half the size of Fab, which may provide increased penetration, but with the offset of less avidity.

Chapter 3

Materials and Methods

3.1 Design of Antibody Fragments

3.1.1 *In silico* humanisation

Using BLASTp, VH and VL sequences of LO1 were interrogated against the human germline VH or VL sequences respectively. This was performed with the CDRs unmasked or masked (the term ‘X’ was input for the CDR residues). Next, the germline sequence was chosen based on the E-value (Expect value)¹. J genes were manually aligned for both VH and VL chains. A homology model was generated using Molecular Operating Environment (Chemical Computing Group, Montreal, Canada) and used to aid in the selection of potential human to mouse framework backmutations. Using a curated list of backmutations from a proprietary database, backmutations that were postulated to have an effect on antigen binding by two or more methods were included as “first-round” backmutations. Those postulated to have an effect on antigen binding by only one method were left as “second-round” backmutations.

IMGT terminology was used when identifying germline sequences most similar to LO1.

¹The E-value “describes the number of hits one can expect to see by chance when searching a database of particular size.”

IMGT germline identifiers are in the following form, IGjtf-g*aa (eg. IGKV1- 10*01); ‘j’ denotes the locus (K, Kappa in this case), ‘t’ denotes the type of gene segment (Variable, V in this case), ‘f’ denotes the gene family (1 in this case), ‘g’ denotes to the gene (10 in this case) and ‘aa’ denotes the allele (01 in this case) (Giudicelli et al. 2004).

3.1.2 *In silico* developability risk profiling

Deamidation

Two proprietary machine learning structure-based GSK algorithms were utilized for deamidation risk prediction. A residue was recorded as a predicted deamidation site when identified by both algorithms.

Aspartate isomerisation

A single proprietary machine learning structure-based GSK algorithm was utilized for aspartate isomerization prediction.

Oxidation

Methionine oxidation sites were predicted within the Fv region using two machine learning structure-based GSK proprietary algorithms. Oxidation sites predicted by both methods were reported as ‘strong’, while sites predicted by only one method were reported as ‘weak’ predictions.

Regular expression motifs

Potential sites for N-glycosylation were investigated by searching for the motif consisting of asparagine followed by any amino acid except proline, either serine or threonine, and finally, again, any residue except proline (N-X-S/T-X).

The presence of non-enzymatic cleavage sites were investigated by searching for the lysine-threonine (KT) and aspartic acid-proline (DP) motifs within the Fv fragment of the sequence.

The Fv sequence was investigated for unpaired cysteines by performing counts of cysteine residues. The presence of a glutamine at the N-terminus was also investigated by searching the Fv sequence.

Immunogenic potential

An immunogenicity risk score was calculated based on the prediction of foreign MHC class II epitopes using a GSK proprietary algorithm ranked against a test set of antibodies with a known immunogenicity response in a clinical setting. A threshold of below 125, 195 units corresponded to low and moderate immunogenicity, based on immunogenicity response in a clinical setting.

Similarity to human sequence analysis

Using the H-score server (<http://www.bioinf.org.uk/abs/shab/>), the Fv HC or LC sequence was entered and searched against the human heavy chain or light chain sequences, respectively. The method for determining the score is described in detail in Abhinandan & Martin (2007).

Prediction of packing angles

Using the Packing Angle Prediction Server (<http://www.bioinf.org.uk/abs/paps/>) the Fv of the HC and LC sequence were entered. The method identifies potential interface residues using a neural network trained on a dataset of 561 antibodies. Interface residues are defined as those positions where solvent accessibility changes as a result of VH/VL interaction. The method for determining the packing angle is described in detail in Abhinandan et al. (2010).

3.2 Cloning of Antibody Fragments

The starting material consisted of plasmids of a chimeric LO1-Fab. The LC was in plasmid pTT5-LO1-LC and HC in plasmid pDOM40-LO1-HC-Cys.

3.2.1 Cloning of murine scFv.

The multiple cloning site (MCS) of pTT5-LO1-LC plasmid was excised by digestion with AgeI and EcoRI and the vector was dephosphorylated using Calf Intestinal Alkaline Phosphatase (CIP) (cat no: M0290: New England Biolabs, Massachusetts, USA) and purified using NucleoSpin Gel and Polymerase Chain Reaction (PCR) Clean up kit (cat no: 12303368, Machrey-Nagel, Duren, Germany).

3.2.2 Cloning of humanised Fab.

pTT5-LO1-LC plasmid was digested with BsiWI-HF and AgeI-HF (cat no: R3553S, R3552S, New England Biolabs, Massachusetts, USA), and for the HC pDOM40-LO1-HC-Cys plasmid was digested with BamHI-HF and NheI-HF cat no: R3136S, R3131S, New England Biolabs, Massachusetts, USA), dephosphorylated using CIP (cat no: M0290; New England Biolabs, Massachusetts, United States) and purified using NucleoSpin Gel and PCR Clean up kit (cat no: 12303368, Machrey-Nagel, Duren, Germany). Infusion reactions were performed using synthetic geneblocks (Appendix 1), appropriately digested vector and 5x Infusion Cloning Mastermix (cat no: 638920, Takara Bio Inc, Shiga, Japan) according to manufacturer's instructions. DH5 α cells (cat no: C2987U, New England Biolabs, Massachusetts, United States) were transformed with infusion mix and grown out in 400 μ l of Super Optimal Broth with Catabolite repression (SOC) media for 45 mins on a shaking platform (37°C, 135 rpm) after which they were plated on 100 μ g/ml ampicillin agar plates and grown overnight in 37°C incubator.

Sanger sequencing was performed by GeneWiz using the following primers. For pTT5 forward

primer: 5'-TGCGCTAAGATTGTCAGTTT-3', reverse primer: 3'-ATTTGTCCTCCAGATCGCAGCA-5'.

3.3 Expression of antibody fragments

3.3.1 Transient transfection

HEK293/6E cells were seeded at a density of 1.75×10^6 cells ml^{-1} in Freestyle293 media (cat no: 12338026, Invitrogen, California, USA) supplemented with $500 \mu\text{g ml}^{-1}$ of Genecitin (cat no: 10131027, Invitrogen, California, USA) or in Expi293 media (cat no: A1435102, Invitrogen, California, USA) with no supplement. $20 \mu\text{g}$ of constructs were added to 2mls of Opti-Mem (cat no: 31985062, Invitrogen, California, USA) along with $80 \mu\text{l}$ of proprietary lipid-mediated transfection reagent and incubated for 15-minutes at room temperature. The DNA:lipid-based transfection complex was added to the cells and grown in Corning Erlenmeyer Shake flasks (cat no: 431255, Corning, New York, USA). Flasks were kept at 37°C in a 5% CO_2 incubator for 7 days. Cells were harvested by centrifugation for one hour at $931 \times g$. Transfections at a larger scale were performed using the same ratio of materials as above. For chimeric Fab expression, a 1:1 ratio of HC to LC ratio was used. The HC to LC ratio was explored for the humanised Fab.

3.4 Purification of Antibody Fragments

3.4.1 Histidine-tagged purification of scFv fragments

Following harvest as described in Methods 3.3.1 supernatant was filtered using $0.22 \mu\text{m}$ Stericup filter units (Merck Millipore, Massachusetts, USA). 2ml of Ni Sepharose resin (cat no: 17526801, GE Healthcare, Chicago, Illinois) was added to Poly-Prep Chromatography Columns (cat no: 7311550, Bio-Rad, California, USA) column and washed with 10 Column volumes (CVs)

of Dulbecco's Phosphate Buffered Saline (DPBS)(cat no: D8537, Sigma-Aldrich, Missouri, USA). Buffer-exchanged supernatant was added to an equilibrated column and flow-through collected. Column was washed with DPBS, 50mM imidazole for removal of non-specifically bound proteins. Protein was eluted with 200mM imidazole.

Supernatant was concentrated and buffer exchanged into PBS using tangential flow filtration (cat no: CD010T010407, Pall Corporation, New York, USA). 2mls of Ni Sepharose Excel resin (cat no: 17371201, GE Healthcare, Chicago, Illinois) was added to Poly-Prep Chromatography Columns (cat no: 7311550, Bio-Rad, California, USA) column and washed with 10x column volumes of PBS. Buffer-exchanged supernatant was added to an equilibrated column and flow-through collected. Column was washed with DPBS, 10mM imidazole (cat no:56749, Sigma-Aldrich, Missouri, USA) for removal of non-specifically bound proteins. Protein was eluted with 20mM imidazole.

HisPur Ni-NTA Resin (cat no: 88221, ThermoScientific, California, USA) was utilised in a similar manner. The column was packed with 1ml of resin. Equilibration was performed in 20mM imidazole after which sample was added and 40mls of 20mM imidazole was added. Sample was eluted using 5mls of 400mM imidazole.

3.4.2 Capto-L purification

Following harvesting and sterile filtration as described in Methods 3.2.1. Poly-Prep chromatography columns (cat no: 7311550, Bio-Rad, California, USA) were packed with Capto-L resin (cat no: 17547801, GE Healthcare, Chicago, Illinois). The resin was washed with 10 CV of DPBS (cat no: D8537, Sigma-Aldrich, Missouri, USA) and equilibrated with 3 CVs of load buffer (55mM Tris, 45mM acetic acid, pH 7.5). Sample was applied to the column and flow-through collected. The column was washed with 5 CVs of detergent containing buffer with no salt (55mM Tris, 45mM acetic acid, 0.3M sodium acetate, 0.1M sodium caprylate, pH 7.5) and re-equilibrated with 3 CVs of load buffer prior to elution using 300mM acetic acid, neutralized with appropriate volume of 1M Tris-HCl. The column was stripped using 0.1M

NaOH and resin stored in 20% ethanol.

For volumes larger than 100mls this was performed using the AKTA Pure System (cat no: 29018224, GE Healthcare, Chicago, Illinois) using 1ml min⁻¹ flow rate and 5ml HiTrap Protein L column (cat no:17547815, GE Healthcare, Chicago, Illinois).

3.4.3 CaptureSelect™ Biotin Anti-IgG-CH1 purification

Culture supernatant was incubated with CaptureSelect™ Biotin Anti-IgG-CH1 Conjugate at a ratio of 10:1 for 2 hours on a rotator (cat no: 11496548, FisherScientific, Massachusetts, USA) at RT. Poly-Prep chromatography columns (cat no: 7311550, Bio-Rad, California, USA) were prepared by washing the column with 30mL of DPBS (cat no: D8537, Sigma-Aldrich, Missouri, USA). The culture supernatant and beads mixture was added to the column and flow-through collected. The antibody was eluted using 100mM glycine with a pH of 2.5 and neutralized with 1M Tris with a pH of 8.8 at a ratio of 5:1. Zeba™ Desalting columns (cat no: 89889, ThermoFisher Scientific, Massachusetts, USA) were used for buffer exchange to PBS.

LO1 was commercially produced from hybridoma stocks, and provided sterile in PBS. It was previously generated and purified as described in Chang et al. (2012), Khamis et al. (2016). Mouse myeloma IgG3κ (cat no: I3784, Sigma-Aldrich, Missouri, USA) was used as a control for LO1.

3.4.4 Size exclusion chromatography

The protein size and oligomerization state were determined by high performance liquid chromatography (HPLC) using a TSKgel G2000SWX1, 7.8 x 300mm (cat no: 808540, Merck, New Jersey, USA) at room temperature. The system was calibrated with a proprietary molecular mass standard. Mobile phase buffer was 100mM sodium phosphate (monobasic), 200mM NaCl, pH6.8, 5% n-Propanol at a flow rate of 0.5ml min⁻¹. Elution was monitored at 280 nm.

Preparative gel filtration was performed using a HiLoad 16/600 prep grade Superdex 75 column (cat no: 28989333, GE Healthcare, Chicago, USA) on an AKTA Pure System (cat no: 29018224, GE Healthcare, Chicago, Illinois) at room temperature. The mobile phase was DPBS (cat no: D8537, Sigma-Aldrich, Missouri, USA) at a rate of 1 ml min⁻¹. Elution was monitored at 280nm. The column was pre-soaked overnight in 1M NaOH to minimize endotoxin contamination.

3.4.5 Cation exchange chromatography

SP Sepharose Fast Flow cation exchange chromatography resin (cat no: 17072904, GE Healthcare, Illinois, USA) was added to polypropylene columns. The resin was equilibrated with 15mls of 50mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid(HEPES), 20mM imidazole, pH 7.5. Elution was performed with 10mls of 50mM HEPES, 20mM imidazole and 1M NaCl. Eluate was directly added to equilibrated His-NTA column and purified as previously described.

3.5 Characterisation of antibody fragments

3.5.1 SDS- Polyacrylamide Gel Electrophoresis

Samples were loaded onto a NuPAGE Novex 4-12% Bis-Tris Mini Protein Gel (cat no: NP0322PK2, ThermoFisher Scientific, Massachusetts, USA) with a total protein concentration of 10 μ g and run using NuPAGE MES Running Buffer (cat no: NP0002, ThermoFisher Scientific, Massachusetts, USA) for 35 minutes at 200v in a XCell SureLock™ Mini-Cell (cat no: EI0001, ThermoFisher Scientific, Massachusetts, USA). Standard protein ladder run on every gel is SeeBlue Plus2 Protein Standard (cat no: LC5925, ThermoFisher Scientific, Massachusetts, USA). The gel was stained with InstantBlue Protein Stain (cat no: ISB1L, Expedeon, Cambridge, UK) according to the manufacturer's instructions and imaged using the Gel Doc-It Imaging System (UVP, Jena, Germany).

For near-infrared gel imaging, Chameleon Duo Pre-stained Protein Standard (cat no: 92860000, Li-Cor Biosciences, Nebraska, USA) was used as the standard and imaged on Odyssey CLx (Li-Cor Biosciences, Nebraska USA) at 700 and 800nm.

3.5.2 Western blotting

Supernatant was either non-reduced or reduced (Dithiothreitol, 95°C boiling for 5 minutes). SDS-PAGE was performed as described in Methods 3.4.3 without staining. The gel was removed from the gel cast and transferred using iBlot™ Transfer Stack (cat no: IB401032, ThermoFisher Scientific, Massachusetts, USA) and iBlot™ Dry Blotting System (cat no: IB1001 ThermoFisher Scientific, Massachusetts, USA) on program P3. Membranes were blocked for 1 hour in 3% Milk (cat no: 70166, Sigma-Aldrich, Missouri, USA) on a Stuart™ See-Saw Rocker (cat no: 10470655, Fisher Scientific, New Hampshire, USA) washed 3 times in 0.05% PBS-Tween20 (PBST). Membranes were incubated overnight at 4°C in 3% milk in anti-HIS (1:1000, cat no: 05-949, Millipore, Massachusetts, USA). The following morning, membranes were washed 3x in 0.05% and incubated with anti-mouse IgG Peroxidase (1:5000, cat no: A9044, Sigma-Aldrich, Missouri, USA) for 45-minutes. Following another 3 washes in PBST, the membrane was developed with SuperSignal™ West Pico PLUS Chemiluminescent Substrate (cat no: 34577, ThermoFisher Scientific, Massachusetts, USA).

3.5.3 Peptide-mass fingerprinting

Protein was run on a 12% Bis-Tris Mini Protein Gel (cat no: NP0341PK2 ThermoFisher Scientific, Massachusetts, USA) with the same settings as for SDS-PAGE described above. The following protocol was performed by Dr Satty Borman, GSK. Protein bands were excised from the polyacrylamide gel, reduced with dithiothreitol, the cysteine residues carboxyamidated and digested in situ with trypsin and chymotrypsin as described by Shevchenko et al. (1996), Wilm et al. (1996).

Preparation of Sample for MALDI-TOF MS Analysis. A 1 μ l aliquot of the liquid surrounding the gel pieces was mixed with 1 μ l matrix solution (60% (v/v) acetonitrile, 0.5% (v/v) trifluoroacetic acid, 6mg/ml α -cyano-4-hydroxycinnamic acid) in the bottom of a microfuge tube. Immediately, 1 μ l of the mixture was spotted onto a 384-well stainless steel MALDI-TOF mass spectrometer target and allowed to dry, leaving a crystallized mixture of sample and matrix on the target for analysis. Peptide calibration standard solution (Bruker Daltonics, Bremen, Germany) was spotted in a similar manner adjacent to the samples.

MALDI-TOF Mass Spectrometry. Matrix-assisted laser-desorption/ionization time-of-flight mass spectra were collected on an Ultraflex Extreme mass spectrometer (Bruker Daltonics, Bremen, Germany) in reflectron mode. Individual peptide ions were selected for fragmentation analysis by LIFT-MS/MS sequencing (Suckau et al. 2003). Spectra were interpreted using FlexAnalysis and Biotools software (Bruker Daltonics) and the data searched against protein sequence databases using the program Mascot (Perkins et al. 1999).

LC-ESI Mass Spectrometry. Digest (5 μ l) was diluted with 5 μ l 0.1% formic acid and 5 μ l injected onto a EASY-nanoLC 1000 coupled to an Orbitrap mass spectrometer (Thermo Scientific). The sample was separated on a reversed-phase Acclaim PepMap RSLC 50 μ m x 15cm, C18, 2 μ m, 100A column using a 1h gradient. Blank injections were 2 μ l.

3.5.4 Immunohistochemistry

NIRF IHC of LO1

Frozen sections of LDLR^{-/-} mouse aortic root were provided by Dr Joseph Boyle (NHLI, Imperial College). Sections were equilibrated in Double-distilled water (ddH₂O) and fixed in 100% isopropanol for one hour. Slides were briefly dipped in ddH₂O to wash and then air-dried for 30 minutes and blocked with 2% BSA (2% Bovine serum albumin (BSA) in PBS, 0.22 μ m sterile-filtered) at 4°C for one hour. Washing steps were conducted with PBS using a dip-wash-dip method, where fresh PBS was used in each instance with a five-minute washing

step. Sections were stained with LO1-750 and IgG3k at 10 μ g/ml in 0.5% BSA and incubated at 4°C overnight. Sections were washed in PBS using dip-wash-dip method and nuclear staining was performed using DAPI (ThermoFisher, UK) at 1:500 in PBS for 10 minutes at RT in the dark. The slides were washed using dip-wash-dip method and air-dried for one hour in a dark container at 4°C. Glass coverslips were mounted using Flouromount G (ThermoFisher, UK) and sections were imaged using the Leica SP5 MP inverted confocal microscope Facility for Imaging by Light Microscopy, Imperial College (FILM). The 633nm laser intensity was set at 93% or above for imaging.

NIRF IHC of LO1-Fab variants

Sections stained with LO1-chFab, LO1-huFab, LO1-controlFab were fixed and blocked in BSA as described above. Subsequently, these slides were blocked using Avidin/Biotin Blocking Kit (Vector Laboratories, USA) according to manufacturer's instructions and stained overnight at 4°C with a concentration of 12.5 μ g/ml in 0.5% BSA. Sections were then washed in PBS using dip-wash-dip method and incubated for an hour at RT with CaptureSelect™ Biotin Anti-IgG-CH1 Conjugate (1:1000, cat no: 11496548, FisherScientific, Massachusetts, USA) in PBS. Sections were washed in PBS using dip-wash-dip method and incubated for an hour at RT with Streptavidin-AlexaFlour647 (ThermoFisher Scientific, Massachusetts, USA). Sections were washed in PBS using dip-wash-dip method and nuclear staining was performed using DAPI (ThermoFisher Scientific, Massachusetts, USA) at 1:500 in PBS for 10 minutes at RT in the dark. The slides were washed using dip-wash-dip method and air-dried for one hour in a dark container at 4°C. Glass coverslips were mounted using Flouromount G (ThermoFisher Scientific, Massachusetts, USA) and imaged on Leica SP5 MP inverted confocal microscope (FILM). The 633nm laser intensity was set at 93% or above for imaging.

NIRF IHC of LO1-scFv

Sections stained with LO1-scFv and control LO1-scFv followed the protocol of the Fab. However, the secondary antibody was 6x-His Tag Monoclonal Antibody (HIS.H8), Alexa Fluor 647 (cat no: MA1-21315-A647, Invitrogen, California, USA).

3.5.5 Oil Red O staining

En face sections of aorta were blocked in 3% BSA for 1 hour. Subsequently, the tissue was stained with Oil Red O (Lipid Stain) (cat no: ab150678, Abcam, Cambridge, UK) according to the manufacturer's instructions.

3.5.6 Modification of LDL

Preparation of MDA was carried out as described by Palinski et al. (1989). Briefly, MDA was produced through the rapid acid hydrolysis of 1,1,3,3-tetramethoxypropane (Malonaldehyde bis-dimethyl acetal, Sigma-Aldrich, Missouri, USA) with 4M of HCl at RT for 10 minutes. The solution was adjusted to a pH of 7.4 using 1.5M NaOH creating a stock solution of 0.5M MDA. Human plasma LDL (cat no: 437644, Merck Millipore, Massachusetts, United States) was incubated with MDA for three hours at 37°C at a ratio of 100 μ l of 0.5MDA to 1mg of LDL. Zeba™ Desalting columns (cat no: 89889, ThermoFisher Scientific, Massachusetts, USA) were used for buffer exchange into PBS. The concentration of MDA-LDL was probed with the use of Pierce BCA Protein Assay Kit (cat no: 23225, ThermoFisher Scientific, Massachusetts, USA) according to manufacturer's instructions.

3.5.7 Fluorescence labelling of antibodies

The labelling of antibodies was undertaken using VivoTag-S 750 MAL (cat no: NEV11223, PerkinElmer, Massachusetts, USA) through the use of maleimide chemistry. LO1 was con-

centrated to 2mg ml⁻¹ using Amicon Centrifugal Filters (cat no: C86533, Merck Millipore, Massachusetts, USA). A 10-20mM stock of VivoTag-S-750 MAL was prepared in dimethyl sulfoxide (DMSO, cat no: D8418, Sigma-Aldrich, Missouri, USA) and added to 2mg of LO1 such that the molar ratio of protein to dye was 1:10. The reaction tube was covered with aluminum foil and rotated for two-hours at RT. Zeba™ Desalting columns (cat no: 89889, ThermoFisher Scientific, Massachusetts, USA) were used for buffer exchange to PBS. The concentration of LO1-Fab was probed using a NanoDrop 2000 spectrophotometer (cat no: ND-2000, ThermoFisher Scientific, Massachusetts, USA). The degree of labelling was determined by measuring the maximum absorbance of the labelled antibody at 750nm and 280nm and using the equations below.

$$Degree of labelling = \frac{[dye]}{[protein]} \quad (3.1)$$

$$[protein] = \frac{A_{280} - (A_{750} * correction factor)}{\epsilon_{protein}} \quad (3.2)$$

$$[dye] = \frac{A_{750}}{\epsilon_{dye}} \quad (3.3)$$

3.5.8 Indirect Enzyme-linked Immunosorbent Assay

Nunc Maxisorp plates (cat no: 44-2404-21, ThermoFisher Scientific, Massachusetts, USA) were coated with 10μg ml⁻¹ of MDA-LDL or 2μg ml⁻¹ of H3 diluted in PBS overnight at RT. Plates were washed three times in PBST (0.05% Tween 20 in PBS) to remove non-adherent material. Plates were subsequently blocked with 3% BSA (3% BSA in PBS, cat no: A9418, Sigma-Aldrich, Missouri, USA) for one hour. The wash process was repeated. Purified mAb or culture supernatant was appropriately diluted in assay buffer (0.5% BSA, 0.05% Tween 20 in PBS) and added to appropriate wells for one hour at RT. The wash process was carried out and plates were incubated with CaptureSelect™ Biotin Anti-IgG-CH1 Conjugate (1:1000, cat no:

11496548, FisherScientific, Massachusetts, USA) for one hour for LO1-Fab samples. The wash process was repeated. The plate was incubated with Streptavidin-Horseradish Peroxidase (cat no: DY998, R&D Systems, Minnesota, USA) at a 1:200 dilution in assay buffer for 20-minutes. The wash process was repeated and 3,3',5,5'-Tetramethylbenzidine (TMB, Sigma-Aldrich, Missouri, USA) was added for 7-minutes after which the reaction was stopped using 0.5M H₂SO₄ and the absorption was measured at 450nm on a plate reader (Beckman-Coulter, California, USA). All steps were carried out at RT using 100 μ l per volume per well. Values were normalised to the averages of blank wells that was run on every plate and received all reagents except for the purified mAb or culture supernatant. All measurements were carried out in duplicates.

For scFv ELISAs, the same protocol as above was followed, apart from the primary antibody being Anti-6X His tag antibody (HRP) (1:5000, cat no: ab1187, Abcam, Cambridge, UK) for one hour.

For LO1 ELISAs, the same protocol as above was followed, apart from the primary antibody being Polyclonal goat anti-mouse IgG Biotin (1:5000, cat no: 103005, Southern Biotech, Alabama, USA) for one hour.

3.5.9 Sandwich enzyme-linked Immunosorbent Assay

Nunc Maxisorp plates (cat no: 44-2404-21, ThermoFisher Scientific, Massachusetts, USA) were coated with 3.12×10^{-7} mols of LO1 and IgG3k or 6.25×10^{-7} mols of LO1-huFab or controlFab overnight at RT. Wells were washed in PBST to remove non-adherent material. Plates were subsequently blocked with 3% BSA, cat no: A9418, Sigma-Aldrich, Missouri, USA, 3% BSA in PBS) for one hour. The wash process was repeated. MDA-LDL was added to the wells 30ug ml⁻¹ in two-fold dilution series for one hour. The wash process was carried out and plates were incubated with Goat pAb Anti-ApoB Biotinylated (1:2000, cat no: ab20898, Abcam, Cambridge, UK). The plate was incubated with Streptavidin-Horseradish Peroxidase (cat no: DY998, R&DSYSTEMS, Minnesota, USA) at a 1:200 dilution in assay buffer for 20-minutes. The wash process was repeated and TMB (Sigma-Aldrich, Missouri, USA) was added for

seven minutes after which the reaction was stopped using 0.5M H₂SO₄ and the absorption was measured at 450nm on a plate reader (Beckman-Coulter, California, USA). All steps were carried out at RT using 100 μ l per volume per well. All measurements were carried out in duplicates.

3.5.10 Competitive enzyme-linked Immunosorbent Assay

Nunc Maxisorp plates (cat no: 44-2404-21, ThermoFisher Scientific, Massachusetts, USA) were coated with 10 μ g ml⁻¹ MDA-LDL overnight. Wells were washed three times in PBST to remove non-adherent material. Plates were subsequently blocked with 3% BSA, cat no: A9418, Sigma-Aldrich, Missouri, USA, 3% BSA in PBS) for one hour. The wash process was repeated. HuFab was added to the wells at a concentration of 25 ug/ml in the presence of various concentrations of competitor for one hour. The wash process was carried out and plates were incubated with CaptureSelect™ Biotin Anti-IgG-CH1 Conjugate (1:1000, cat no: 11496548, FisherScientific, Massachusetts, USA) for one hour. The plate was incubated with Streptavidin-Horseradish Peroxidase (cat no: DY998, R&D Systems, Minnesota, USA) at a 1:200 dilution in assay buffer for 20-minutes. The wash process was repeated and TMB (Sigma-Aldrich, Missouri, USA) was added for seven minutes after which the reaction was stopped using 0.5M H₂SO₄ and the absorption was measured at 450nm on a plate reader (Beckman-Coulter, California, USA). All steps were carried out at RT using 100 μ l per volume per well. All measurements were carried out in duplicates.

3.6 *In vivo* characterisation of antibody fragments

3.6.1 Ethical approvals

The methods were carried out in accordance with the approved guidelines. All animal experimental protocols undertaken at Imperial College were ethically approved by Imperial College and were carried out under the authority of UK Home Office licenses. All animal studies

comply with our collaborator's policy - GSK Policy "Care, Welfare and Treatment of Animals".

3.6.2 Mouse model of Atherosclerosis

Pathogen-free female LDLR^{-/-} mice were obtained from Jackson Laboratories (cat no: 002207, Maine, USA) and housed in a controlled environment under specific pathogen-free conditions. To initiate the onset of disease, the mice were fed a high-fat Western diet (1% CO, 40.5% Suc, 0.25% cholesterol, TD 190191; Envigo, USA) from the age of 10 weeks until experimental age. Unless otherwise stated, age matched C57BL/6 were used as Wild-type (WT) controls for all studies and were maintained on standard chow (RM3; SDS Diets, UK).

3.6.3 Half-life determination of fragments

C57/BL6 mice were injected with 50 μ g of huFab-750 or 50 μ g of LO1-scFv-750 via a tail intravenous injection. Vena section was performed on the opposite tail vein and 20 μ l of blood was immediately mixed with 20 μ l heparinised saline. Samples were taken at 0, 0.5, 3, 4, 24, 48, 72 and 144 hours post injection. Samples were blank subtracted from mouse blood without any fluorescent probe delivered in triplicate on the Odyssey CLx (Li-Cor Biosciences, Nebraska, USA) at 800nm.

3.6.4 Fluorescence Molecular Tomography Imaging and Computed Tomography (FMT/CT)

Agents targeted at specific biomarkers of atherosclerotic disease were imaged using FMT quantitative tomography according to manufacturer's instructions. The FMT setup is shown in Figure 3.1A. IntegriSense-680 (PerkinElmer, Massachusetts, USA) was administered at dose of 2nmol/25g/mouse. MMPsense-645 FAST (PerkinElmer, Massachusetts, USA) was administered at a dose of 4nmol/25g/mouse. HuFab was administered at a dose of 1nmol/25g/mouse (2mg/kg).

Fluorescent agents were administered intravenously 4-hours prior to imaging.

Prior to imaging, mice were anesthetized using isoflurane (5% for induction and 1.5% for maintenance in 100% Oxygen with a flow rate of 1.5 L/min) and fur was removed using a shaver followed by Nair Sensitive depilatory cream (Church & Dwight Co, USA) and rinsed off with warm water. The anesthetized mice were carefully positioned in the imaging cassette during scans. Transillumination settings were set at very high sensitivity for MMPSense-645 FAST and HuFab and normal sensitivity for IntegriSense-680. Fluorescence reflectance imaging (FRI) was routinely performed prior to each tomographic imaging session with built-in LED illuminators. FMT Imaging experiments were conducted at the Biological Imaging Centre, Imperial College, London using FMT 4000 (PerkinElmer, Massachusetts, USA).

Once FMT scanning was finished the cassette with the mouse was transferred to a custom CT-compatible mouse bed. The bed was connected to isoflurane to maintain anesthesia. Finally, the animal was centered in the CT field of view using laser alignment to cover the whole heart. CT acquisition was performed using 180 projections over the 360 degrees of rotation. Each projection lasted for 200 ms of exposure time with 80 V tube voltage and 500 μ A tube current. X-ray detector binning was set to 4 $\times$ 4, which conferred an isotropic spatial resolution of 103 μ m. In order to reduce the radiation exposure to the animals and facilitate follow up scanning, the scanner mode was converted from conventional helical scanning to step and shoot mode with a settle time of 50ms (this conversion also limited the vibration induced by the rotation of the gantry, hence minimizing motion blurring artifacts). The scan time for each animal was approximately 10 minutes. The CT scan projections were reconstructed using Feldkamp algorithm using Shepp Logan filter with no down sampling. CT Imaging experiments were conducted at the Biological Imaging Centre, Imperial College, London using Siemens Inveon PET/CT (Siemens Medical Solutions USA, Inc) with the help of Dr Ali Ashek, Imperial College London.

Omnipaque 300 (GE Healthcare, Illinois, USA) was delivered at a dose of 1.5g/iodine/kg weight for contrast-enhanced high resolution CT to localize the aortic root and major arteries

in the neck and thorax to guide interpretation. Data was exported as Digital Imaging and Communications in Medicine (DICOM) for both CT and FMT and colocalization performed using fiducial markers (Figure 3.1B) and AMIDE (SourceForge).

Fluorescence data was reconstructed by TrueQuant 3.0 (PerkinElmer, Massachusetts, USA) software and used for the quantification of three-dimensional fluorescence signal within the aortic arch region. No fluorescence thresholding was applied to the tomographic datasets. For organ distribution studies, tissues were harvested and imaged on an opaque tissue block as described above.

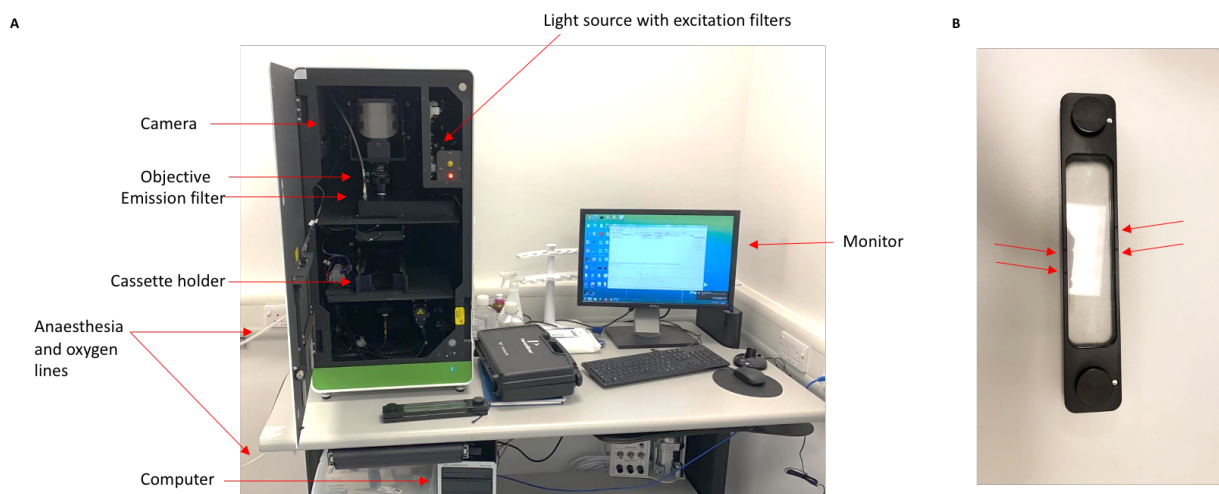


Figure 3.1: Setup of FMT system. A) FMT setup with key components of the system identified with a red arrow. B) Cassette where a mouse or tissue sample is placed into for FMT imaging. Red arrows indicate fiducial markers on cassette.

3.6.5 *Ex vivo* tissue imaging

Resected vessels were briefly rinsed in DPBS to remove free blood. The vessel was placed on cassette and imaged using epifluorescence settings at 645nm, 680nm and 750nm corresponding to the probes MMPSense, IntegriSense, and HuFab, respectively using the FMT 4000 (PerkinElmer, Massachusetts, USA).

3.7 Statistical Analysis

For comparisons between two groups with Gaussian distribution, a student's T-test was performed. The data was tested for normality, if it was normally distributed Welch's correction was performed otherwise a Mann-Whitney correction. Statistical tests were calculated using Prism 8 (GraphPad, California, USA). Data is presented mean $\pm$ Standard error of the mean (SEM) and statistical significance was achieved when $p < 0.05$.

For *in vitro* studies, coefficient of variation calculations were performed and maintained at below 10% to ensure replicability of data. For *in vivo* studies, power calculations were performed to ensure a group size of 4-6 would have 90% power assuming 5% significance and a two-sided test. Power calculations were performed on the basis of the data from Khamis et al. (2016).

Chapter 4

Humanisation of a LO1 Fragment Antigen Binding (Fab)

4.1 Introduction

The identification of LO1 from a female hyperlipidemic mouse gives LO1 murine origins. Thus, it is likely to elicit an immune response in humans. Humanisation of antibodies has become a key step in the preclinical phase of the development of therapeutic antibodies since HAMA responses were observed in response to muromonab (Orthoclone OKT3). There is a multitude of humanisation techniques today as reviewed in Chapter 2 (Literature Review). The majority of techniques humanise only the variable regions of the antibody with the constant regions being taken from human germline sequences. In this thesis, the approach of “CDR grafting” is taken for the humanisation of LO1, whereby the murine CDRs of LO1 are grafted onto a human framework scaffold. In this chapter, I present the design, expression and purification of humanised Fabs of LO1 and characterise them both *in vitro* and *ex vivo*. The *in silico* design was performed with the help of Dr Alan Lewis and his team at GSK. The peptide mass fingerprinting was performed with the help of Dr Satty Borman, GSK.

4.2 Results and Discussion

4.2.1 *In Silico* Analysis of Sequence and Structure

Basic Local Alignment Search Tool (BLAST) finds regions of local similarity between sequences thereby aiding the determination of the most similar human germline sequence to that of murine LO1 (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). The expect value (E value) is the number of hits expected to be seen by chance given the size of the database being investigated. Therefore, low E-values (less than 0.01) indicate an evolutionary relationship between the compared sequences (Pearson 1998). This analysis was performed for the VL and VH independently, with their CDRs either included or masked out. The placeholder 'X' was utilised when masking out CDR residues during the alignment. BLAST data presented in this thesis was accessed in November 2017, unless otherwise stated.

Variable light chain

Murine sequences of the LO1 VL were evaluated with BLAST protein (BLASTP) to investigate the most similar human germline sequence to LO1. To evaluate whether the CDRs influenced the top hit human germline sequence, alignments were performed with (unmasked) and without the CDRs (masked). IGKV1-39 was identified as the first and second hit when CDRs were unmasked and masked, respectively. The remaining top five hits were different between unmasked and masked, indicating the CDRs played a role in the most similar germline sequence (Table 4.1). These data in conjunction with GSK proprietary gene frequency information resulted in IGKV1-39 being chosen as the VL gene template. J genes were aligned manually with LO1 where IGKJ4*01 was selected due to the number and position of sequence differences being distal to the CDRs (Figure 4.1).

Table 4.1: Human light chain V-gene germlines similar to LO1 V-gene. Performed using BLASTP with CDRs unmasked and masked. Top hits of sequences producing significant alignments. Accessed November 2017.

(a) With CDRs.		(b) With CDRs masked.	
Germline	E value	Germline	E value
IGKV1-39	6.00 x 10 ⁻³⁴	IGKV1D-16	1.00 x 10 ⁻²⁶
IGKV3D-7	1.00 x 10 ⁻³³	IGKV1-39	2.00 x 10 ⁻²⁶
IGKV3-7	5.00 x 10 ⁻³³	IGKV1-6	2.00 x 10 ⁻²⁶
IGKV1-8	9.00 x 10 ⁻³³	IGKV1D-12	2.00 x 10 ⁻²⁶
IGKV4-1	1.00 x 10 ⁻³²	IGKV1-12	2.00 x 10 ⁻²⁶
IGKV1D-16	1.00 x 10 ⁻³²	IGKV1-33	3.00 x 10 ⁻²⁶
IGKV3-11	2.00 x 10 ⁻³²	IGKV1-27	3.00 x 10 ⁻²⁶
IGKV3D-15	3.00 x 10 ⁻³²	IGKV1-13	5.00 x 10 ⁻²⁶
IGKV3-15	3.00 x 10 ⁻³²	IGKV1OR2_108_ORF_ONL	6.00 x 10 ⁻²⁶
IGKV1-6	3.00 x 10 ⁻³²	IGKV4-1	8.00 x 10 ⁻²⁶

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LO1      FGGGTKLDIK
IGKJ1*01 -----VE--
IGKJ2*01 -----E--
IGKJ3*01 --P---V---
IGKJ4*01 -----VE--
IGKJ5*01 -----R-E--

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Figure 4.1: J-gene alignment of LO1 light chain.

Variable heavy chain

Similar to the light chain, the murine sequence of LO1 VH was evaluated using BLASTP to investigate the most similar human VH germline sequence to LO1. Again, this was performed with and without the CDR residues to evaluate whether the CDRs played a role in identifying the most similar germline sequence. IGHV1-46, IGHV1-2 and IGHV1-69 were all in the top five hit germline sequences for masked and unmasked CDRs alignments. While IGHV1-46 ranked higher in both lists (2nd on both lists) with the help of proprietary GSK gene frequency information, IGHV-69 was chosen as the VH gene template (Figure 4.2). J genes were aligned manually with LO1 and IGH6*01 selected due to the number and position of sequence differences being distal to the CDRs (Figure 4.2).

GSK proprietary gene frequency information indicated that the pairing of IGKV1-39 and

IGHV1-69 was a natural pairing. The light chain IGKV1-39 and heavy chain IGHV1_69 featured in 14.25% and 6.17% of the antibodies in a database of well-expressed and somatically rearranged antibodies.

Table 4.2: Human heavy chain V-gene germlines similar to LO1 V-gene. Performed using BLASTP with CDRs unmasked and masked. Top hits of sequences producing significant alignments. Accessed November 2017.

(a) With CDRs.		(b) With CDRs masked.	
Germline	E value	Germline	E value
IGHV1_2	9.00×10^{-40}	IGHV1_69	8.00×10^{-30}
IGHV1_46	1.00×10^{-39}	IGHV1_46	1.00×10^{-29}
IGHV1_8	1.00×10^{-37}	IGHV1_18	3.00×10^{-29}
IGHV1_69	1.00×10^{-37}	IGHV1_3	7.00×10^{-29}
IGHV1_3	3.00×10^{-37}	IGHV1_2	9.00×10^{-29}
IGHV1_18	9.00×10^{-37}	IGHV1_8	3.00×10^{-28}
IGHV5_51	5.00×10^{-35}	IGHV1_45	6.00×10^{-28}
IGHV1_45	7.00×10^{-35}	IGHV1_24	2.00×10^{-27}
IGHV1_58	3.00×10^{-34}	IGHV1_58	5.00×10^{-27}
IGHV1_24	1.00×10^{-33}	IGHV5_51	1.00×10^{-26}

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LO1      WGQGTTL----
IGHJ1*01 -----LV----
IGHJ2*01 --R--LV----
IGHJ3*01 -----MV----
IGHJ4*01 -----LV----
IGHJ5*01 -----LV----
IGHJ6*01 -----V----

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Figure 4.2: J-gene alignment of LO1 heavy chain.

Site-specific cysteinylolation for conjugation

Past work from our group has demonstrated a loss of binding of LO1 to MDA-LDL upon conjugation of the antibody using N-hydroxysuccinimide (NHS) chemistry on lysine residues. This abrogation of binding has not been observed during conjugation of the antibody using maleimide chemistry (Figure 4.3A,B). This has led to the hypothesis that lysines in LO1 are crucial for binding to antigen. Within the variable regions of LO1 there are nine lysines in the VH, with two of those in CDR-H2 and another two in CDR-H3. In the VL there are seven lysines with one each in CDR-L1 and CDR-L2 (Figure 4.3C). Thus, to avoid compromising

lysines when conjugating Fab with a fluorophore, an unpaired cysteine was incorporated through the inclusion of the natural IgG hinge region comprising the upper hinge and first cysteine of the core hinge (Figure 4.3D) (Mehanna et al. 2017). The antibody fragment is termed a Fab' due to the inclusion of a thiol functional group (Crivianu-Gaita & Thompson 2016).

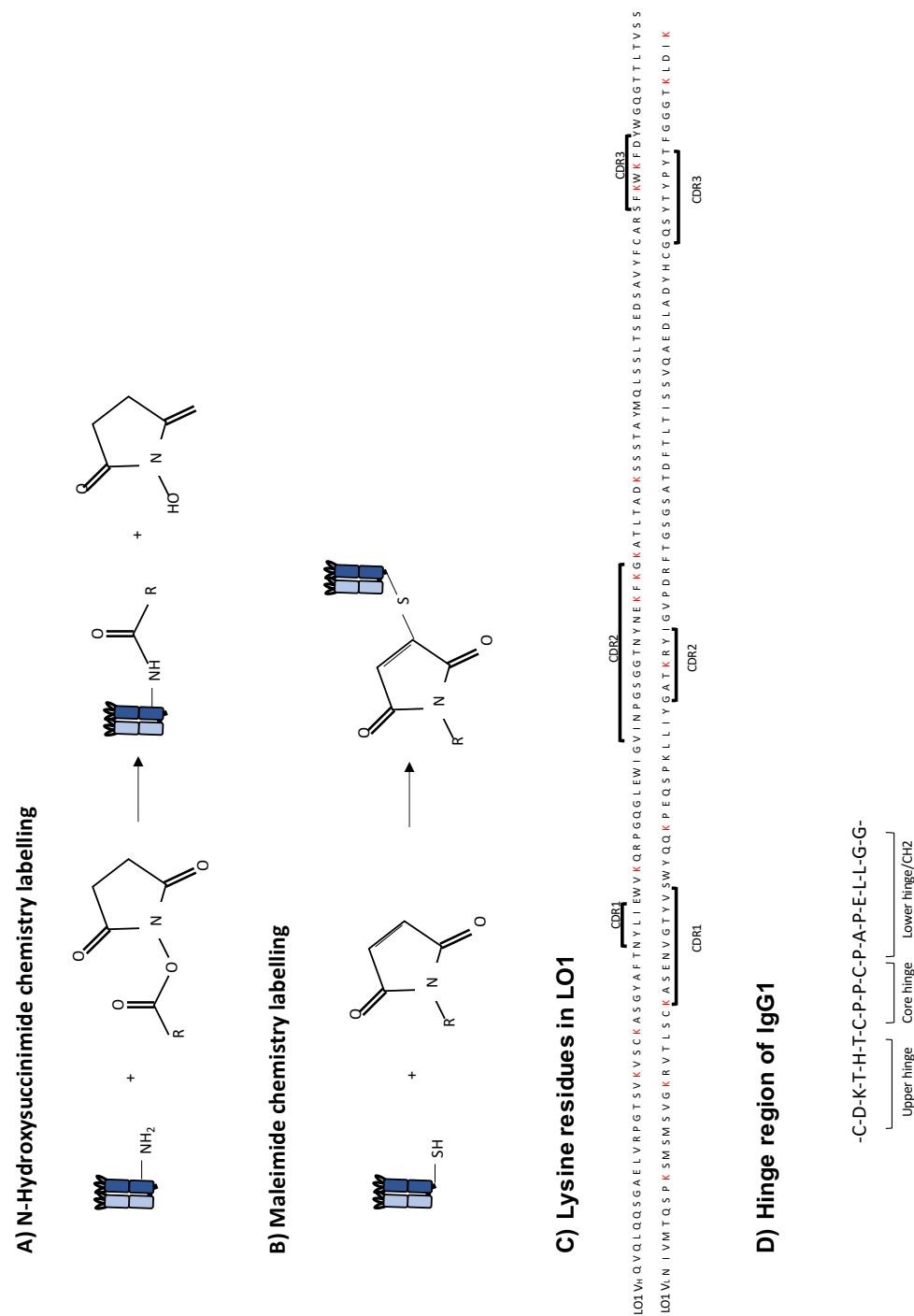


Figure 4.3: Incorporation of a cysteine tag for conjugation with a NIR fluorophore. A) NHS labelling using an amine containing molecule. B) Maleimide labelling using a sulphydryl containing molecule. C) Sequence of murine mAb LO1. CDRs annotated based on Kabat numbering. Lysine residues identified in red. D) Upper hinge of IgG1 and the first cysteine of the core hinge was incorporated into the design of LO1 humanised Fab for site-specific cysteination of fluorophore.

Backmutation analysis

In CDR grafting of a mAb it is common to require framework residues (backmutations) from the donor antibody in place of their orthologues in the acceptor frameworks to restore binding to antigen after humanisation (Foote & Winter 1992). The need to backmutate residues to their murine residues arose as X-ray crystallography evolved and the field came to an understanding that the CDRs were not limited to just the apical loops (in the case of CDR-H1) and can be impacted by residues in the β -sheet itself. Residues previously recognized as interface residues or those important for backmutation were identified using a GSK proprietary database. The residues were segregated depending on whether two or more methods identified them as residues necessary for backmutation (Table 4.3). Those identified by two or more methods were identified as crucial for restoring antigen-binding (first-round backmutations). Those residues identified by only one method were kept as second-round backmutations, only being employed should the first-round fail to restore affinity of the huFab to antigen or if further modification of affinity was required. A homology model was generated for the CDR-grafted humanised LO1 using CCG (Chemical Computing Group) Molecular Operating Environment (MOE) to identify clusters of residues for backmutation on the basis of their side-chain position (Figure 4.4).

Light chain. Four humanised VL constructs with different backmutations were designed *in silico* using the homology model in Figure 4.4A,B. These are numbered L0 to L3 (Figure 4.5). L0 is a CDR graft of LO1 VL CDR residues into the acceptor framework IGKV1_39 using the Kabat CDR definition. L1 is identical to L0 except for a backmutation at position 68 from Gly to Ala. This residue is packed against CDR-L1 thus may affect antigen interaction due to the loss of the side-chain on glycine during humanisation. L2 is identical to L0 except for a backmutation at position 87 from Tyr to His. This interface residue is located in the β -sheet directly prior to the apical loop of CDR-L3 (residues 89-97) and results in a change back to a polar residue from a hydrophobic one. Position 87 is likely to play a role in antigen interaction by changing the orientation of the VH and VL chains in relation to one another. L3 combines the backmutations in L1 and L2 variants.

Residues at positions 1 and 3 were identified by only one method as important for backmutation. Interestingly, asparagine at position 1 is rare in the human germline sequence. If it were necessary to further backmutate these constructs, these two positions should be backmutated together in one variant due to their sequence and structural proximity to one another. Leu78Val is separate to other residues identified for backmutation and should be examined with its own variant chain as shown Figure 4.4. All three of these residues did not meet the criteria for being identified by two or more methods for first round backmutation (Table 4.3).

Heavy chain. Five humanised VH constructs with different backmutations were designed using the homology model in Figure 4.4A,B. H0 is a CDR graft of LO1 VH CDR residues into the acceptor framework IGHV1.69. These are numbered H0 to H4 (Figure 4.5). H1 is identical to H0 except for backmutations at positions 27, 28 and 30. These residues form part of the structural loop of CDR-H1 as defined by Chothia. H2 is identical to H1 except for backmutations at positions 48, 67 and 69. Met48Ile is located directly prior to CDR-H2 while Val67Ala and Ile69Leu are located directly after CDR-H2. These residues are likely to contribute to the structure of the CDR-H2 loop itself and, due to their structural proximity, they are likely to work in a synergistic manner. H3 is identical to H1 except for a backmutation at position 91 from Tyr to Phe. This interface residue is located close to CDR-H3 and is likely to affect the VH-VL orientation (Figure 4.4).

Ser16Thr is distant to the apical CDR loops and Arg66Lys while close to the apical loops did not meet the criteria of being identified by two or more methods for first-round backmutation (Table 4.3).

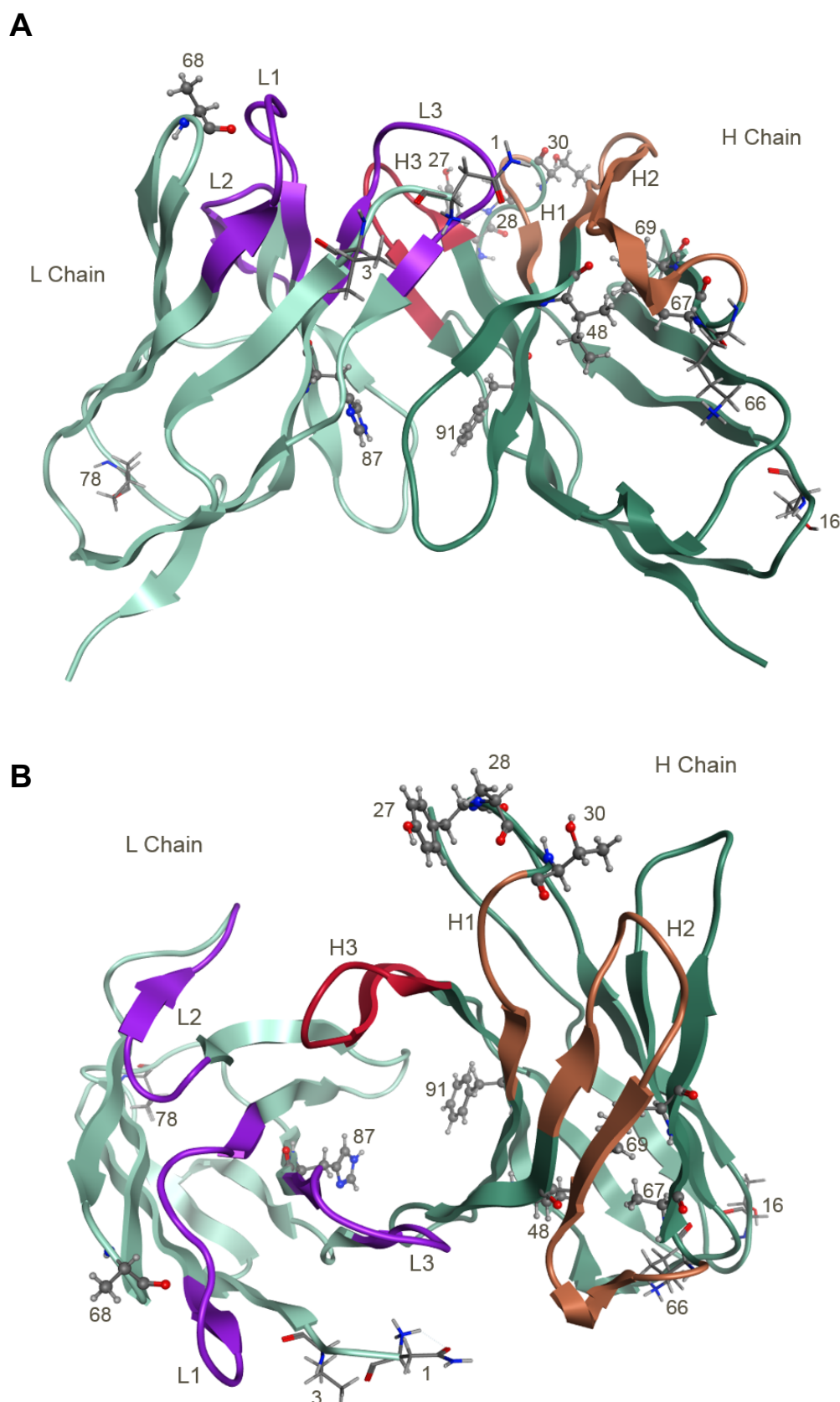


Figure 4.4: Homology model of CDR grafted LO1. A homology model of LO1 was generated using Molecular Operating Environment v2016.0802. This model was used to identify residue clusters based on side chain position and/or direction. A) Side view of CDR-grafted LO1. B) Top-down view of CDR-grafted LO1 looking at the VH-VL interface. CDR-H residues in brown, CDR-H3 residues in red, CDR-L residues in purple.

Kabat number	1	2	3	4	5	6	7	8	9	1
LO1VL	N I V M T Q S P K S M S M S V G K R V T L S C K A S E N V G T V V S W Y Q Q K P E Q S P K L L I Y G A T K R Y I G V P D R F T G S G S A T D F T L T I S S V Q A E D L A D Y H C G Q S Y T Y P Y T F G G G T K L D I K									
L0	D - Q - - - - S - L - A - - - - T I T - - - - K A - - - - - S - F S - - - - G - - - - L - P - - F - - - - V E - -									
L1	D - Q - - - - S - L - A - - - - T I T - - - - K A - - - - - S - F S - - - - A - - - - L - P - - F - - - - V E - -									
L2	D - Q - - - - S - L - A - - - - T I T - - - - K A - - - - - S - F S - - - - G - - - - L - P - - F - - - - H - - - - V E - -									
L3	D - Q - - - - S - L - A - - - - T I T - - - - K A - - - - - S - F S - - - - A - - - - L - P - - F - - - - H - - - - V E - -									
			CDR1			CDR2				CDR3
Kabat number	1	2	3	4	5	6	7	8	9	1
LO1VH	Q V Q L Q Q S G A E L V R P G T S V K V S C K A S G Y A F T N Y L I E M V K O R P G Q G L E W I G V I N P G S G G T N Y N E K F K G K A T L T A D K S S S T A Y M Q L S S L T S E D S A V Y F C A R S F K W K F D Y W G Q G T T L T V S S									
H0	- - - - V - - - - V K K - - - S - - - - - G T - - S - - - - - R - - - - - M - - - - - R V T I - - - - - T - - - - - R - - - - - T - - - - - Y - - - - - V - - - -									
H1	- - - - V - - - - V K K - - - S - - - - - Y A - - T - - - - - Y A - - T - - - - - R - - - - - M - - - - - R V T I - - - - - T - - - - - R - - - - - T - - - - - Y - - - - - V - - - -									
H2	- - - - V - - - - V K K - - - S - - - - - Y A - - T - - - - - Y A - - T - - - - - R - - - - - I - - - - - R T L - - - - - T - - - - - R - - - - - T - - - - - Y - - - - - V - - - -									
H3	- - - - V - - - - V K K - - - S - - - - - Y A - - T - - - - - Y A - - T - - - - - R - - - - - M - - - - - R V T I - - - - - T - - - - - R - - - - - T - - - - - F - - - - - V - - - -									
H4	- - - - V - - - - V K K - - - S - - - - - Y A - - T - - - - - Y A - - T - - - - - R - - - - - I - - - - - R A T L - - - - - T - - - - - R - - - - - T - - - - - F - - - - - V - - - -									
			CDR1			CDR2				CDR3

Figure 4.5: Variants for humanisation of LO1. Four variants of the LC and five variants of the HC were generated incorporating mutations identified as important for maintaining antigen contact. L0 and H0 are direct CDR-graft onto chosen human germline sequences, with successive iterations incorporating different mutations. CDRs annotated based on Kabat definitions (black). CDR-H1 by Chothia definition in blue. Backmutation residues identified in red, backmutation residues that comprise of CDR-H1 by Chothia definition are identified in blue. Standard amino acid nomenclature used.

Table 4.3: Residues identified for backmutation to murine residue. CDR-H1(Chothia definition) backmutations are excluded from table.

	Residues identified by 1 method	Residues identified by ≥ 2 methods
Light chain	D1N	G68A
	Q3V	Y87H
	L78V	
Heavy chain	S16T	M48I
	R66K	V67A
		I69L
		Y91F

4.2.2 *In silico* developability risk profiling

The five VH and four VL humanised variants were combined in an array fashion to yield 20 humanised Fab clones. Homology models of each clone were generated using MOE with a maximum of 10 FR Templates and 10 mainchain models, and model refinements set to fine. During the manufacturing, storage and *in vivo* delivery, antibodies and their subsequent fragments may be at risk for degradation or altered properties through various pathways (Eon-Duval et al. 2012). Deamidation, oxidation and asparagine isomerisation sites were evaluated through structural analysis. Immunogenicity, cleavage sites and N-glycosylation sites were evaluated through a sequence-based analysis. The structure-based and sequence-based motif analyses were performed for the Fv region only, whilst the immunogenicity analyses were run on the whole molecule. Developability risks of the IgG1 C-region are well understood and therefore analyses are focused on the Fv region. PTMs of proteins are used to expand the chemical space of proteins greater than the limit imposed by the encoded amino acids. However, the presence of these may lead to a heterogenous preparation of biological drugs and thus their characterization is crucial for regulatory compliance and reliability (Miksinski & Shapiro 2012).

Structure-based analysis

Deamidation. Deamidation is a critical quality attribute in mAbs that has been reported to affect both *in vitro* and *in vivo* stability (Eon-Duval et al. 2012, Beck et al. 2013). For example,

progress in developing protein-based anthrax vaccines has been halted by the instability resulting from asparagine deamidation in anthrax protective antigens (Verma et al. 2013, D’Souza et al. 2013). Furthermore, *in vivo* deamidation of a humanised IgG1 in CDR-H2 reduced its efficacy (Vlasak et al. 2009). Apart from this, deamidation may also impact immunogenicity as it could potentially create epitopes that may be recognised as foreign by the immune system.

The spontaneous non-enzymatic conversion of asparagine to aspartic acid via the deamidation pathway introduces a negative charge which may alter protein function or its susceptibility to degradation (Yang & Zubarev 2010). The potential for deamidation sites within the Fv region of the 20 humanised Fab clones was investigated using two proprietary machine learning structure-based GSK algorithms. A residue was recorded as a predicted deamidation site when identified by both algorithms. No deamidation risk was identified for the Fv region or specifically for any CDR residues (Figure 4.6). Asparagine residues are present only within the CDRs in the humanised clones. A structure based analysis is preferential when analysing deamidation risk as a sequence-based approach generally relies only on the identification of the asparagine-glycine motif, which can result in an over-engineering of antibodies (Delmar et al. 2019).

Aspartate isomerisation. Aspartate isomerisation can lead to altered susceptibility to proteolysis, trigger autoimmune responses, and a loss of its therapeutic activity (Cacia et al. 1996). Reduced binding affinity has been observed upon Asp isomerisation in CDR-H3 (Wakankar et al. 2007). The conversion of aspartate to isoAsp adds an extra methylene residue to the protein backbone which can lead to reorientation of side chains affecting binding (Verma et al. 2016, Cacia et al. 1996). Aspartate isomerisation was investigated using a single proprietary structure-based GSK algorithm based on the deamidation algorithms discussed. No clones or CDRs were predicted to be at risk of aspartate isomerisation (Figure 4.6). The presence of glycine or serine in the n+1 position has been found to provide the most favourable conditions for isomerisation when in a loop structure. This is likely due to these residues being highly flexible (Sydow et al. 2014). While an aspartate is present at position 101 in CDR-H3 it is not followed by glycine or serine providing a flexible environment to allow isomerisation.

Oxidation. Protein oxidation can occur during the production of antibodies. While methionine oxidation is most reported and researched, other amino acids including tryptophan, cysteine, lysine and histidine have been reported to be oxidised (Dashivets et al. 2016, Ji et al. 2009). There is evidence that oxidation within the CDR residues may reduce or completely abolish binding to antigen by antibodies (Hensel et al. 2011). However, much of the focus of oxidation is for a full mAb and the effect on interactions with FcRn (Bertolotti-Ciarlet et al. 2009).

Methionine oxidation sites were predicted within the Fv region using two machine learning structure-based GSK proprietary algorithms. Oxidation sites predicted by both methods were reported as 'strong', while sites predicted by only one method were reported as 'weak' predictions. Both the Fv and CDRs alone demonstrated no risk of oxidation (Figure 4.6). The humanisation process itself may have reduced the oxidation risk as it resulted in two methionine residues (VL, residue 11 and 13) being exchanged for alternative residues during grafting. One methionine residue remains on each chain in positions of low solvent accessibility (VL - residue 4, VH - residue 80)(Figure 4.5).

It is important to note that two tryptophan residues are present in CDR-H3. Residues within the CDR are more susceptible to oxidation because of the flexible solvent accessible formation of the loops. Pavon et al. (2019) identified that tryptophans in CDR-H3 can be oxidised and correlate with their surface accessible area. Additionally, oxidation of tryptophan in CDR-H3 in a humanised antibody has been demonstrated to result in loss of binding (Wei et al. 2007). Without experimental studies it would be difficult to ascertain whether these residues are a cause for concern as tryptophan oxidation was not predicted in this study.

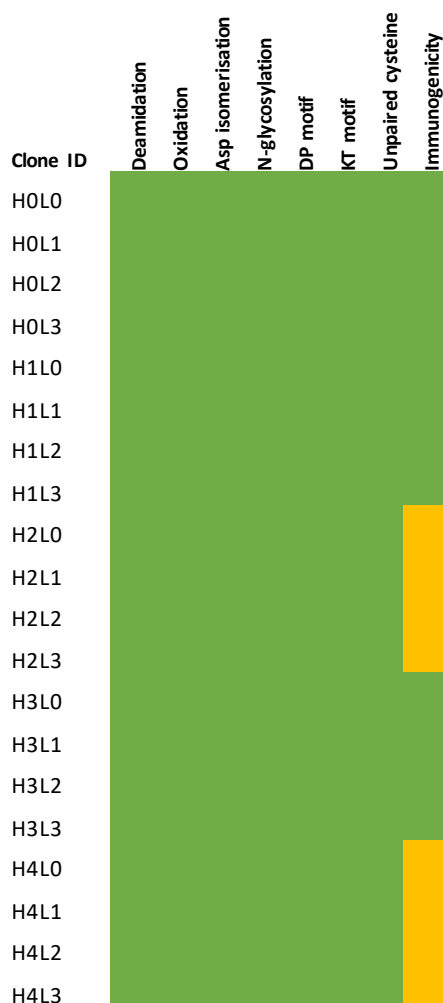


Figure 4.6: *In silico* analysis of developability of humanised variants. Green, little to no risk; Yellow, medium risk; red, high risk.

Sequence-based analysis

Regular expression motifs. Recent evidence has demonstrated the importance of Fab glycosylation on stability, binding characteristics and affinity (Schneider et al. 2015, Goletz et al. 2012). Potential sites for N-glycosylation were investigated by searching for the motif consisting of asparagine followed by any amino acid except proline, either serine or threonine, and finally, again, any residue except proline (N-X-S/T-X) (Y & G 1990). The motif was not present in either the murine LO1 or humanised variants (Figure 4.6 and 4.5).

Fragmentation of mAbs is another critical quality attribute which should be monitored to assess the integrity of the protein. It can occur during protein production and develop during

storage and *in vivo* delivery. Non-enzymatic cleavage sites determined by the lysine-threonine (KT) and aspartic acid-proline (DP) motif were investigated. Under mildly acidic conditions, aspartic acid followed by another residue is one of the most frequent degradation pathways, and of these the aspartic acid-proline bond fragmentation is the fastest (Marcus 1985, Li et al. 2009).

A KT cleavage site is present in the humanised variants of LO1 due to the incorporation of the upper hinge region for site-specific cysteination. This KT cleavage site has been previously described in the hinge region of human IgG1 (Smith et al. 1996). Apart from this site, KT and DP motifs were not observed in any of the humanised variants (Figure 4.6 and 4.5). However, fragmentation is dependent on the conditions in which the protein is kept, such as pH, temperature and solvent composition. Differences in these from tested conditions, may lead to increased fragmentation.

The occurrence of glutamine at the N-terminus of the LC and/or the HC is a common occurrence. In addition, the cyclisation of these N-terminal glutamines to pyroglutamate is common as well. This results in loss of 17 daltons. All five VH humanised variants have glutamine on the N-terminus of the HC and thus are susceptible to its cyclisation. The presence of this modification in endogenous human IgG and the observation of negligible effect on antibody structure (Yu et al. 2006), antigen binding (Lyubarskaya et al. 2006) or *in vivo* clearance (Liu et al. 2011), means that the presence of this N-terminal modification is likely to have a minimal effect on function.

As mentioned, unpaired or extra cysteine residues can result in heterogeneity, misfolding or aggregation of the antibody. Thus, the presence of these residues was manually investigated. Extra cysteine residues were not present apart from the engineered cysteine for conjugation to fluorophore .

Immunogenicity. The aim of humanising antibodies is to reduce the immunogenic potential allowing them to be safely administered as diagnostics and therapeutics. An immunogenicity risk score was calculated based on the prediction of foreign MHC class II epitopes using a GSK proprietary algorithm ranked against a test set of antibodies with a known immunogenicity

response in a clinical setting. A threshold of less than 125 and 195 foreign MHC class II epitopes corresponded to low and moderate immunogenicity respectively, based on immunogenicity response in a clinical setting.

The humanised clones utilising H2 and H4, which incorporated the Val67Ala and Ile69Leu backmutations demonstrated a moderate risk of immunogenicity with a combined score of 128 foreign MHC class II epitopes recognised between their respective VH and VLs. The remaining variants showed little to no risk of immunogenicity with H0 and H1 backbone variants having a predicted score of 119 and 120, respectively (Figure 4.6). While these numbers of T-cell epitopes may seem large, the numbers were compared against the numbers of T-cell epitopes of other clinically-validated antibodies and their immunogenicity scores. It is also common for *in silico* tools to be over-predictive (Kuramochi et al. 2019).

The question when assessing “humanness” of an antibody sequence is how representative the antibody sequence is of the expressed human repertoire. There are various measures of humanness. Abhinandan & Martin (2007) described a method to define the “humanness” of an antibody variable sequence based on the mean sequence identity and its standard deviation against a human data set giving a value of the “degree of humanness” known as the z-score. In Figure 4.7A,C, it is clear that as the LC goes sequentially from L0 to L3 the z-score moves closer to the LO1-murine z-score and towards the peak distribution of murine light chain sequences. Interestingly, for the HC z-scores the H2 and H4 variants were closer to the peak distribution of murine sequences than H3. This closely matches the prediction that H2 and H4 had a higher immunogenic potential due to having higher number of T-cell epitopes (Figure 4.7B,D). While every human antibody is 100% human by nature, the z-score demonstrates the spread of human sequences in our repertoire. This analysis has also demonstrated that only a few residues can vastly change the immunogenic potential of an antibody.

The International Nonproprietary Name (INN) is a generic name given to a drug by the World Health Organisation (WHO). In 2014 the definitions for different categories of antibodies was updated to reflect changes in technologies. The antibodies are now categorised by their sequence

identity rather than the method by which their sequences are obtained. As a consequence, many antibodies that were previously categorised as 'humanised' are now defined as 'mixed'¹ or 'chimeric' (Jones et al. 2016). A threshold of greater than 85% of sequence identity was defined as 'humanised' and below this chimeric. By this definition, H1L0 would be classified as chimeric as its sequence identity to human is VL - 82.1% and VH - 84.7%. Furthermore, the top-hit for the VH is *Macaca fascicularis*, not human. Taking the top-hit rule into account would mean that H1L0 would be classified as chimeric. The new INN designations are reliant on how similar the CDRs are to human CDRs, with those that are dissimilar being unlikely to meet the new definition. The definitions are not rooted in clinical evidence, for example, trastuzumab, a humanised antibody, would be categorised as chimeric by the new definitions despite its low immunogenic profile (Ismael et al. 2012).

¹One chain is humanised and one chain chimeric

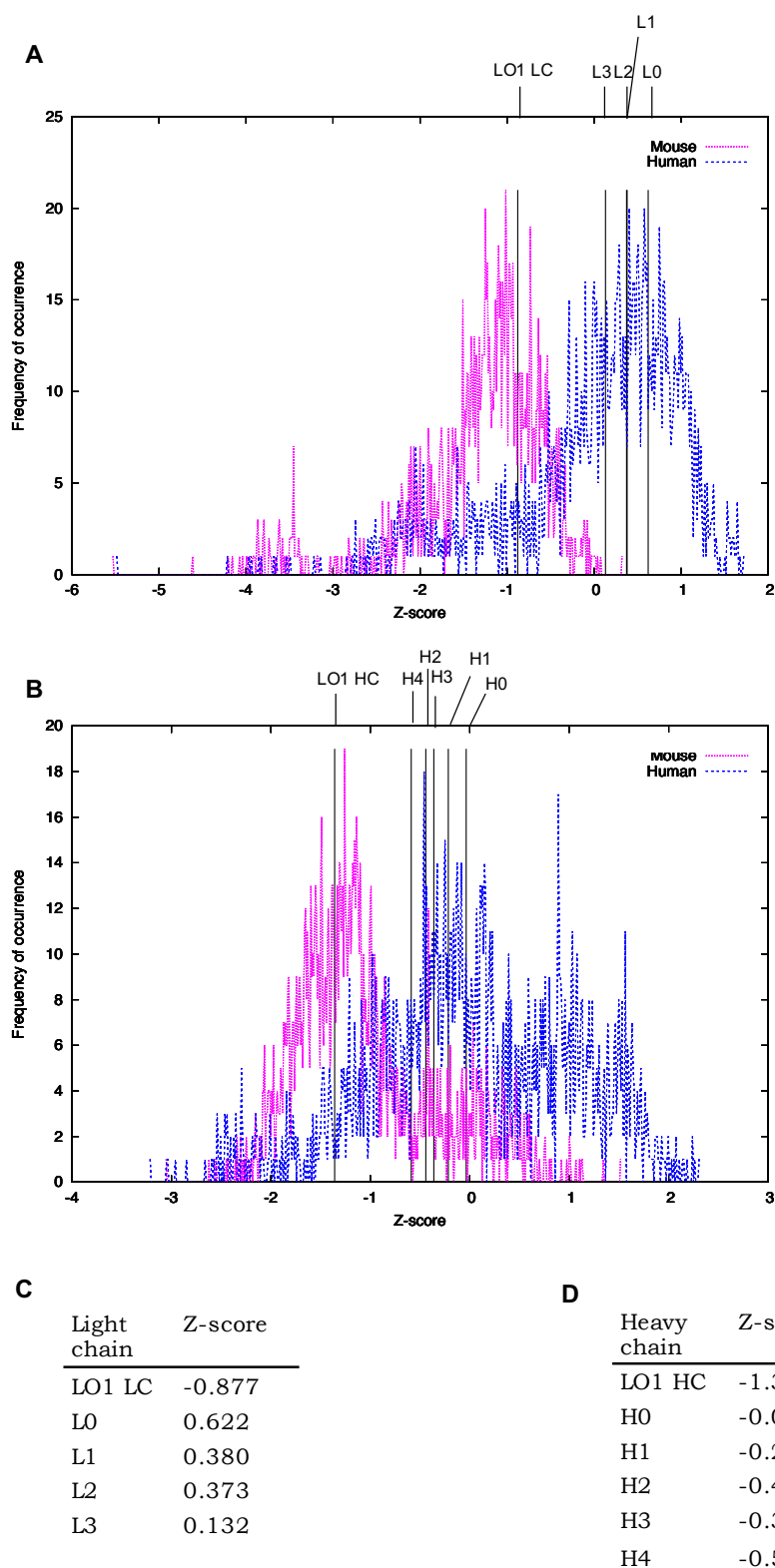


Figure 4.7: Assessing the “humanness” of humanised LO1 variants. A) Light chain z-scores with murine LO1 (mu) and humanised light chains (L0-L3) overlaid. Pink peak represents sample of z-scores of murine antibodies, blue peak represents sample of z-scores of human antibodies. B) Heavy chain z-scores with murine LO1 and humanised heavy chains (H0-H4) overlaid. C) Numerical values of light chain z-scores with murine LO1 and humanised light chains (L0-L3). D) Numerical values of heavy chain z-scores with murine LO1 and humanised heavy chains (H0-H4).

Packing angles. The packing structure of the VH and VL domain can influence the topography of the ABS (Chatellier et al. 1996). This aspect has been largely ignored during the modeling and humanisation process, with a greater appreciation of its importance growing (Abhinandan et al. 2010, Sircar et al. 2009, Marze et al. 2016). For example, the Adair patent (Adair et al. 1994) demonstrated that modification of residue 23 on the VH significantly affects the binding between antibody and antigen. Using Packing Angle Prediction Server (PAPS, <http://www.bioinf.org.uk/abs/paps/>) the torsion angles between the variable regions were predicted for the murine and humanised variants of LO1. The packing angles of the humanised variants differed from the murine; however most remained within the standard deviation of whole PAPS dataset ($-45.6^{\circ} \pm 3.3^{\circ}$)(Table 4.4).

Abhinandan et al. (2010) identified key residues for prediction of the packing angle. These include L42, L87 and H91 which have been affected by the humanisation. In all humanised variants, L42 has been modified from glutamic acid to lysine. In L0 and L1, L87 has been modified from histidine to tyrosine and backmutated in L2 and L3. In H0, H1 and H2, H91 has been grafted from phenylalanine to tyrosine, but backmutated in H3 and H4. Using the PAPS server, the packing angle of each clone was predicted. This revealed that the predicted packing angle is LC dependent in H0, H1 and H2. However, a backmutation in the H3 variant (Phe91Tyr) plays a role in the VH/VL torsion angle as its implementation independently brings the torsion angles closer to the murine LO1 packing angle of -46.4° (Table 4.4). This changed effect on torsion angle is maintained in the H4 variant, which incorporates this same mutation. The residue identified by Adair, H23 is not changed in the humanised variants (Figure 4.5). During lead selection, it may be important to consider these torsion angles as it may affect the topography of the ABS and affect the binding. This may also lead to increased clarity as to the important residues outside of the CDR that play a role in the paratope indirectly by affecting the VH-VL angle.

Table 4.4: Predicted packing angle of murine LO1 and humanised variants using PAPS.

	Packing Angle
Murine LO1	-46.4
H0L0	-45.7
H0L1	-45.7
H0L2	-40.8
H0L3	-40.8
H1L0	-45.7
H1L1	-45.7
H1L2	-40.8
H1L3	-40.8
H2L0	-45.7
H2L1	-45.7
H2L2	-40.8
H2L3	-40.8
H3L0	-46.8
H3L1	-46.8
H3L2	-44.4
H3L3	-44.4
H4L0	-46.8
H4L1	-46.8
H4L2	-44.4
H4L3	-44.4

4.2.3 Expression of an array of humanised Fabs

The expression level can be a measure of variable domain stability and suitability (Ewert et al. 2003). Poor expression may have an impact on the developability of a mAb downstream (Werner 2004, Eon-Duval et al. 2012). The yield for each Fab variant was determined from a normalised expression and purification process. The plasmids containing the HC and LC were co-transfected into HEK293 cells and harvested after 4 days. LO1 huFab variants with the H1 (L0,L1,L2,L3) and H3 (L0,L1,L2,L3) heavy chain backbone had low total expression in the Fab form. This was characterised by densitometry analysis of the ~ 48 kDa band on SDS-PAGE which demonstrated seven out of the eight variants to express 10% or less of Fab (Figure 4.8A). Once the Fab fragment was purified from crude supernatant using Protein L, variants on the H3 backbone demonstrated significantly lower expression levels than Fab fragments expressed with other heavy chain backbones. This is likely to be due to the backmutation as the H3 variant, only had one mutation (Figure 4.8B). This backmutation may have affected the VH-VL

interaction, although from the predicted packing angles this seems unlikely. Instead, this point mutation may have affected the expression or folding of the HC and in turn affected the yield of Fab fragment. It is clear that expression of humanised LO1 using a H0 or H3 variant resulted in low expression of Fab. This in turn resulted in low binding to antigen, likely due to the lack of Fab added into the ELISA from supernatant (Figure 4.8D). Expression using either H1, H2 or H4 backbones gave comparable results in Fab production. Additionally, L2 appears to be well correlated with expression with H1L2 reaching nearly 8mg ml^{-1} (Figure 4.8B). Purification using Capto L (Protein L) demonstrated a contaminant or breakdown product at approximately 11kDa (Figure 4.8C). Similar contaminants have been identified by others (Das et al. 2005). This was later confirmed through peptide mass fingerprinting to likely be a cleavage product of the light chain (Appendix B).

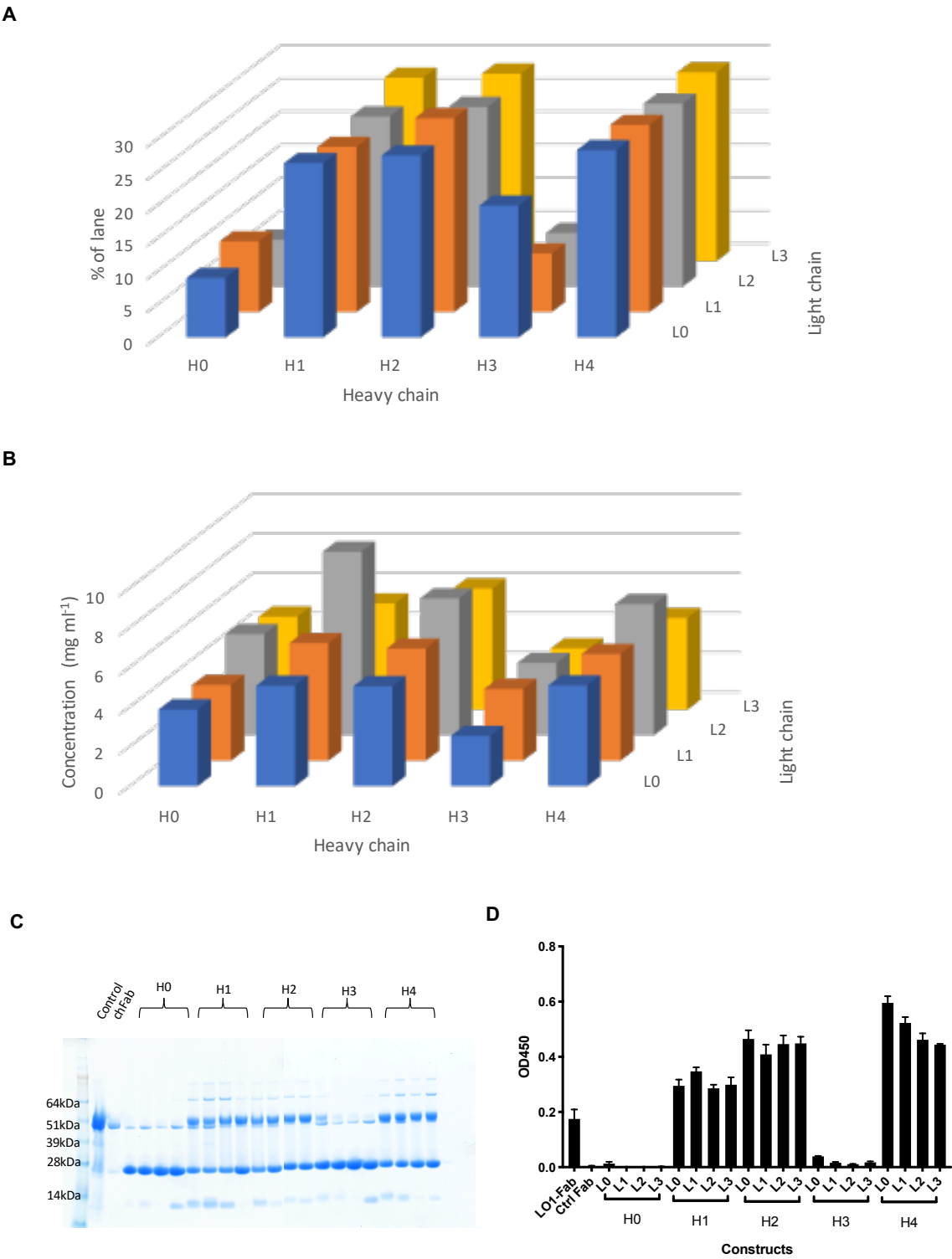


Figure 4.8: Expression yield of humanised Fabs. A) Expression of Fab (47kDa) band as percentage of total expression from SDS-PAGE. B) The concentration (mg ml^{-1}) obtained after purification from protein L from combinatorial transfections of humanised LO1 variants. C) SDS-PAGE of humanised Fab clones purified from protein L. D) ELISA showing reactivity of humanised Fab clones to antigen, MDA-LDL. Values are mean $\pm$ SEM of technical replicates.

Humanised Fab variants which are expressed with H2 and H4 heavy chains are predicted to

have higher immunogenicity due to having more T-cell epitopes (Figure 4.6). These variants demonstrated a slight increase in binding to the antigen on ELISA (Figure 4.8D), however this may be down to variable amounts of Fab due to use of crude supernatant. Furthermore, the variants utilising the H3 heavy chain, showed poor expression likely contributing to its lower reactivity to antigen. As the aim of humanisation was to reduce the immunogenicity of the Fabs, the variants expressed with the H1 heavy chain were chosen for further optimisation due to their lower predicted immunogenicity and good expression profile.

Without a high resolution structure of the antibody variable region of the humanised Fab it would not be possible to tell which residues are involved in antigen interaction. However, the humanisation process has provided some clues through the backmutation process. Residues such as those employed in the backmutation of the H3 variants abrogated expression of the Fab, demonstrating the importance of this residue for its expression.

Optimisation of lead Fab expression and purification

It is common to require optimisation during the expression of proteins in mammalian cells (Vazquez-Lombardi et al. 2018). A strong band of free LC during the expression of the humanised Fabs prompted an investigation into whether optimising the HC:LC could yield greater quantities of Fab with less free LC. Three different ratios of HC:LC were compared, 1:1, 5:1 and 10:1. The higher HC expression reduced the free LC and lower molecular weight contaminants as well as increasing the Fab band (Figure 4.9A). A statistically significant difference in binding of these expressed Fabs was not observed (Figure 4.9B). As there was no difference in binding between the LC variants and expression levels were not a significant concern L0 was chosen for the lowest immunogenic profile. Thus, this led to H1L0 10:1 being selected as the lead humanised Fab clone and results from this clone only will be presented in the remainder of this chapter.

There are many available purification resins for Fab purification. Until this point, all the data presented used Protein L to purify the clones. Protein L captures antibodies on the kappa light chain (Nilson et al. 1992). Protein G is most commonly associated with its antibody binding on

the Fc region of IgG. Despite this, it is capable of binding antibody fragments through a site on the CH1 region (Nezlin 1998). Newer alternatives to these classic proteins are available such as CaptureSelect, utilising a llama HC antibody fragment to interact with human Fab fragments. I investigated whether our clone bound more efficiently to Protein G or CaptureSelect than our current strategy using Protein L. Affinity chromatography purification was performed with these three resins. Purification using CaptureSelect had a clear advantage over both Protein L and G (Figure 4.9C), however total amount of protein purified was comparable at 1.25mg of total protein. Protein G and CaptureSelect both bind a Fab fragment at the CH1 domain while Protein L binds on the VL domain. Thus, differences between CaptureSelect and Protein L may be down to this difference. On the other hand it is not clear as to why protein purified using CaptureSelect bound antigen any differently to Protein G purified protein, especially given total protein yields were comparable. Due to availability and cost of the resins, Protein L was continued to be used due to the volume of Fab required for studies. It may be worth exploring the use of CaptureSelect if costs allow, although benefits may be minor.

LO1-huFab-H1L0 showed bands at 25 and 50kDa in non-reducing SDS-PAGE. A doublet was also observed at around 50kDa in all expressions. To investigate whether this phenomenon was due to disulphide bond formation or glycosylation the clone was incubated with dithiothreitol (DTT) and PNGaseF before being analysed by SDS-PAGE (Figure 4.9D). DTT is a reducing agent that reduces disulphide bonds while PNGaseF is an amidase which cleaves most N-linked oligosaccharides from glycoproteins. As expected the addition of DTT formed a thick band at 25kDa. As predicted from the *in silico* work, the presence of PNGaseF did not show a shift in molecular weight or change in the doublet indicating the doublet was unlikely to be from N-glycosylation. Peptide mass fingerprinting (PMF) revealed three oxidation sites on the HC and one on the LC that were not predicted from the *in silico* work (Appendix B). All four oxidation sites were methionines.

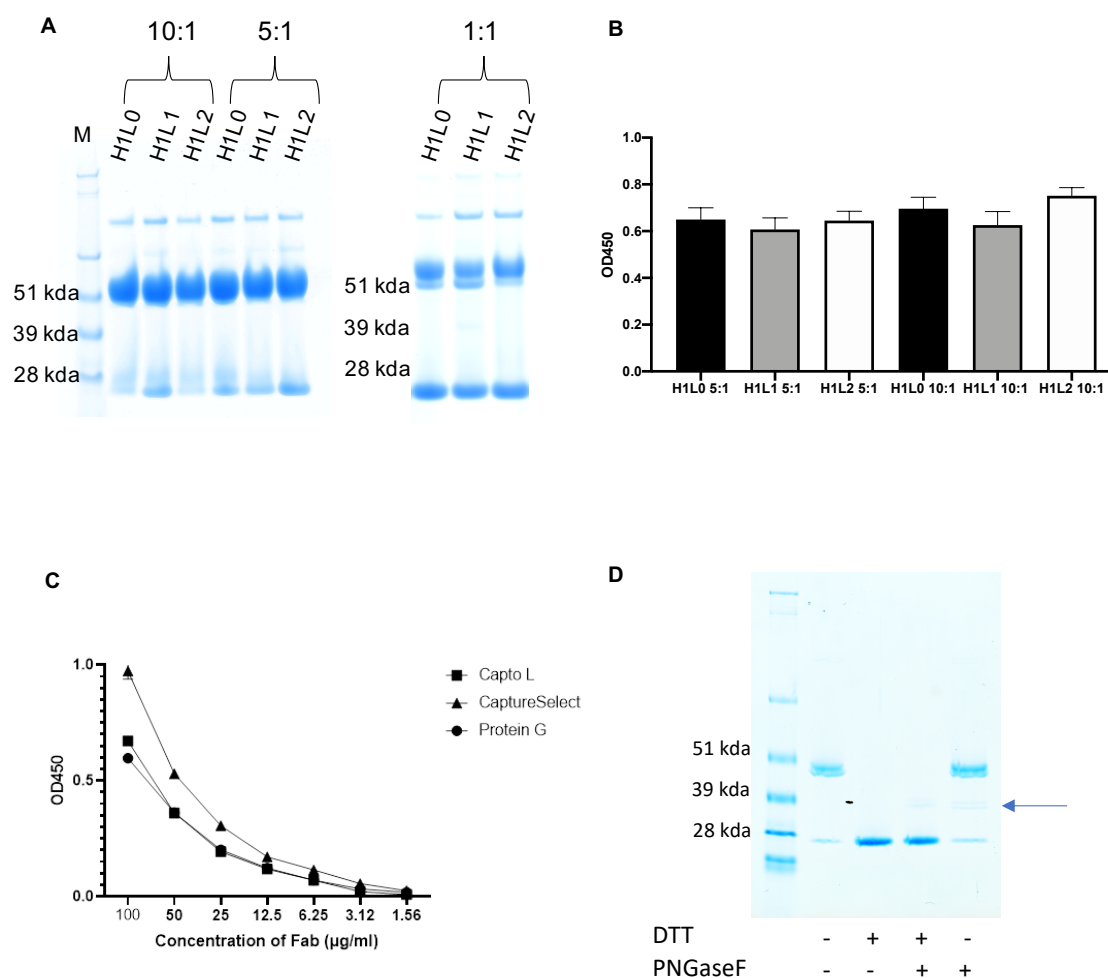


Figure 4.9: Optimisation of expression and purification of Fabs. A) SDS-PAGE of expression of humanised Fab variants with varying HC:LC ratio plasmid transfection. B) Reactivity of different ratios of HC:LC ratio. C) Reactivity of H1L0 using different resins for affinity purification. D) SDS-PAGE of humanised Fab incubated with DTT and PNGaseF. The arrow indicates the band for PNGaseF (36kDa). Values are mean $\pm$ SEM of technical replicates.

4.2.4 Characterisation of the lead humanised Fab

The purity post-affinity purification was determined to be only 21% for band 1 (Figure 4.10A). PMF was performed confirming that band 1 ($\sim 47\text{kDa}$) was a mix of HC and LC, representing the Fab fragment. Band 2 ($\sim 23\text{kDa}$) was identified as predominantly being LC with some HC. In Band 3 ($\sim 12\text{kDa}$) only LC was identified. This is similar to reports by Das et al. (2005) who demonstrated that free LC contaminant at this same molecular weight was a by-product when purifying proteins using Protein L. Size exclusion chromatography (SEC) was employed to remove lower molecular weight proteins and increase the purity of the preparation

(Figure 4.10C). After SEC, only one peak remained for the 50kDa band. Intact mass analysis was performed with the deconvoluted masses shown in Figure 4.10D. The Fab was confirmed as a monomer with a mass of 47412 daltons, matching the theoretical mass of 47319 da. This 93-dalton difference may be due to a PTM. Post-SEC H1L0 was found to bind MDA-LDL and not native LDL (nLDL) (unoxidised).

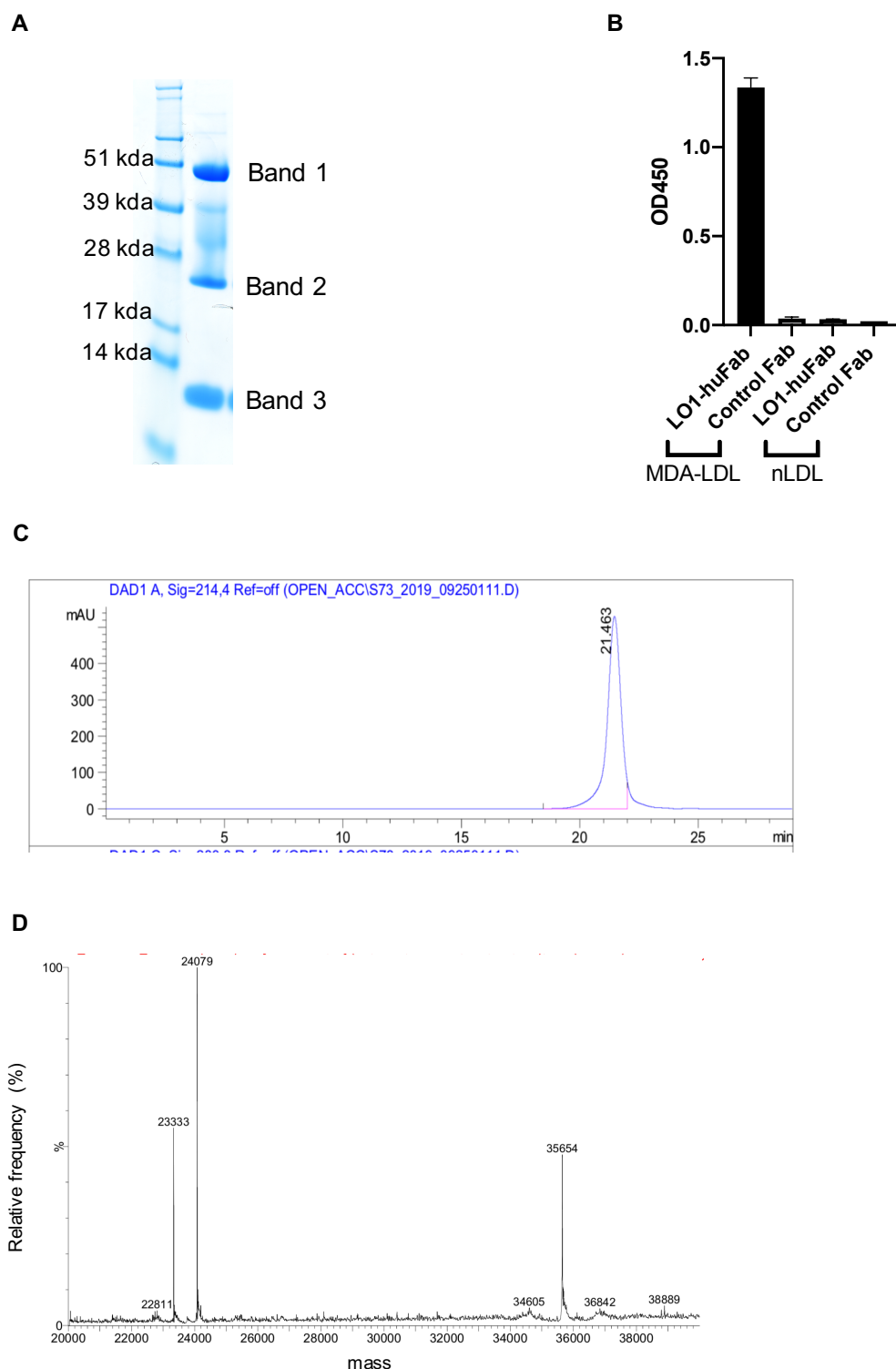


Figure 4.10: Characterisation of a humanised LO1-Fab. A) 12% Bis-Tris SDS-PAGE gel of H1L0 showing purity and used for peptide mass fingerprinting. B) ELISA showing binding of H1L0 (HuFab) against MDA-LDL and native LDL (nLDL). C) Analytical size exclusion chromatography plot after size exclusion chromatography purification showing only one peak. D) De convoluted intact mass spectra of H1L0. B) ELISA showing binding of H1L0 (HuFab) against MDA-LDL and native LDL (nLDL). Values are mean $\pm$ SEM of technical replicates.

4.2.5 Antigen binding of humanised Fabs

The affinity of an antibody or antibody fragment is important as generally only high affinity antibodies are used in clinical work. Using regression analysis the EC₅₀ of H1L0 was estimated to be 177nM (Figure 4.11A, dose response curve not shown). EC₅₀ cannot be directly correlated with affinity as it is not a direct measure of drug affinity. It may suggest that the Fab' affinity is high, similar to the full antibody and could be used therapeutically. However, if the affinity was lower and within the micromolar range designating it to have 'moderate affinity', it would likely still be effective for its intended use. This is because moderate affinity antibodies do not experience the binding site barrier effect and are more likely to diffuse into the tissue as they are released from their first encountered antigens. In order to measure the affinity, the current gold standard of surface plasmon resonance (SPR) was attempted using MDA-LDL to determine kinetic constants. However, MDA-LDL is a large and heterogenous antigen which made it difficult to perform accurate SPR experiments as any chemical modifications (e.g., biotinylation) required to get reliable data would impact the epitope influencing affinity data, so this was not pursued.

A competitive ELISA, where two antibodies compete with one another for binding to antigen can help determine the relative affinities and is routinely used for epitope binning. Antibodies that compete with one another, most likely recognize the same epitope or a nearby one (steric hindrance may play a role in this). Given that the CDRs were maintained during the humanisation, it was expected that the huFab and murine LO1 would compete for the same epitope. This was investigated by adding purified LO1 and huFab H1L0 to a MDA-LDL coated plate at various concentrations. The binding of H1L0 was inhibited by the addition of increasing concentrations of LO1 (Figure 4.11B). This indicated that the huFab had the same epitope on MDA-LDL as the murine LO1.

Although estimated EC₅₀ are also available for the full mAb, due to different assays setups, these are not comparable with one another. In an attempt to compare the full mAb with the humanised Fab, a sandwich ELISA set-up was used. The sandwich ELISA set-up allows the

same secondary antibody (in this case anti-ApoB) to be used on both antibodies, removing the effect of different affinities of secondary antibodies (anti-mouse IgG or anti-CH1) that are present in the indirect ELISA format. The mAb and Fab were coated on solid phase at concentrations of equal molar binding sites and antigen was added in fluid phase. Detection was performed using an anti-apolipoprotein B antibody to measure antigen. This demonstrated that when the antibody formats had equal binding sites, they bound MDA-LDL in a similar fashion (Figure 4.11C). This was important for determining the dose for our *in vivo* studies in the next chapter (Chapter 5).

Finally, prior to beginning *in vivo* studies the labelling process using maleimide chemistry was tested. The advantage of using maleimide chemistry over lysine chemistry is the natural lower prevalence of cysteines within the sequence in comparison to lysine. Furthermore, the naturally occurring cysteines within antibody fragments will form double-bonds preventing labelling of these residues. The engineered unpaired cysteine, thus serves as the only site for conjugation with the fluorophore. A common issue experienced with lysine chemistry labelling is lysine residues forming part of the antigen-binding site. Fortunately, with the extra cysteine being on the C-terminus, it should not distort the tertiary structure of the fragment nor impact its function. This was the case for LO1 Khamis et al. (2016). Lysine chemistry labelling was also performed, which showed highly abrogated binding to MDA-LDL after conjugation (data not shown). There was minimal change in reactivity after cysteine conjugation with a NIR fluorophore (Figure 4.11D).

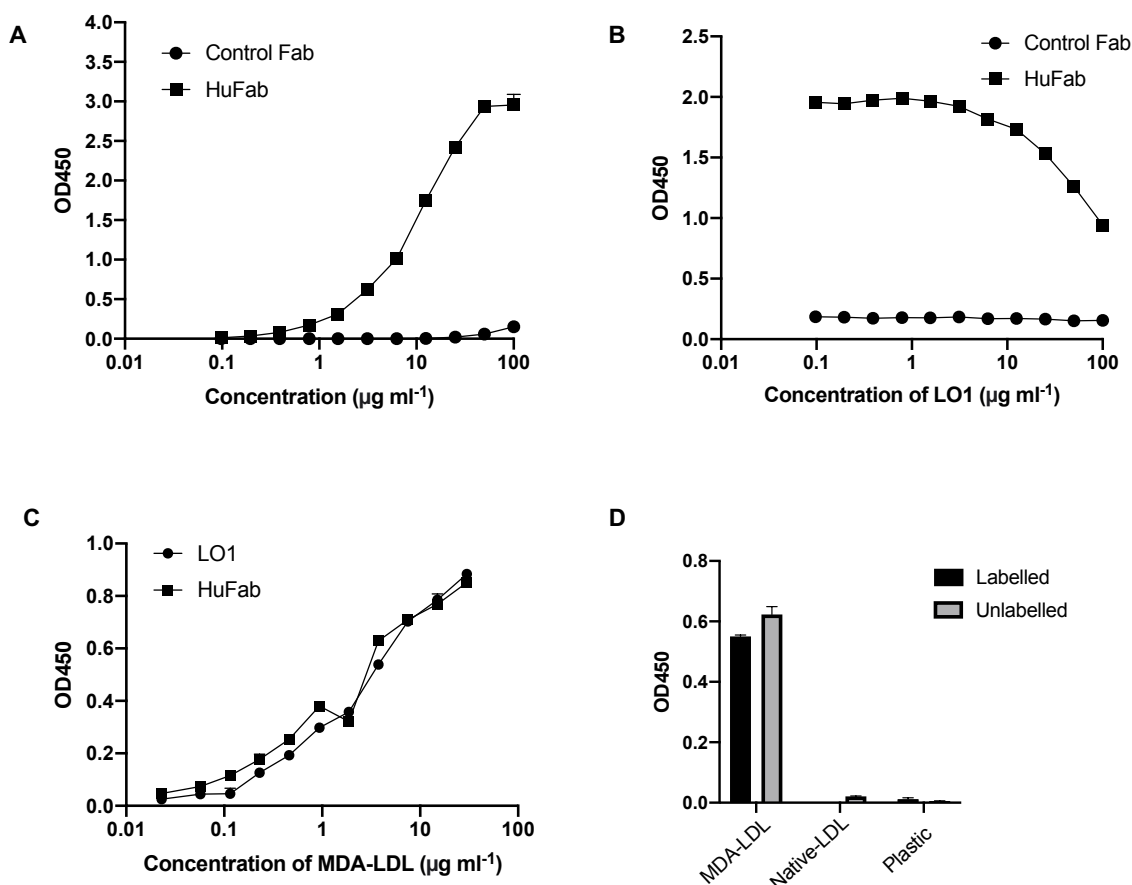


Figure 4.11: Characterisation of a humanised LO1-Fab. A) ELISA showing saturation curve of H1L0 (HuFab) and Control Fab against antigen MDA-LDL. B) Competitive ELISA showing increasing concentrations of LO1 incubated with $12.5 \mu\text{g ml}^{-1}$ of HuFab. C) Sandwich ELISA showing LO1 and HuFab coated at equal . Probed with an anti-ApoB secondary antibody to detect MDA-LDL. D) ELISA showing minimal loss of reactivity after labelling with a NIR fluorophore using maleimide chemistry. The huFab did not bind to native LDL or plastic pre or post-labelling with fluorophore. Values are mean $\pm$ SEM of technical replicates.

4.3 Conclusion

In this chapter, I have described the *in silico* design for humanising LO1 to generate LO1-HuFab. First-round backmutations were instituted to restore binding to antigen. Our initial *in vitro* data indicates that these second-round backmutations were not necessary to employ. However, should further refinements for affinity or stability be warranted these have identified in Table 4.3. The Fabs were expressed in mammalian cells and characterised *in vitro* and using a competition assay shown to bind the same epitope as LO1, the full mAb. Similarly, LO1-huFab reacts only with MDA-LDL and does not bind native LDL or plastic.

Unlike a full mAb, Fabs are not commonly glycosylated and thus can be produced in a variety of expression systems, including gram-negative and gram-positive bacteria or yeast. As part of an increasing demand for animal-free products, alternative production platforms have arisen where proteins are expressed using insect cells, plant cells or cell-free expression systems.

The key advantage of the *E. coli* expression system is that it does not have a long production cycle. This leads to a reduced capital investment when expressing proteins in comparison to mammalian or alternative expression systems. For antibodies and their fragments, including a Fab fragment, the challenge of expressing within *E. coli* is the production of correct disulphide bonds. The bacterial cytoplasm is a reducing environment and thus is not naturally conducive to disulphide bond formation. Using signal sequences expression can be directed to the naturally oxidative periplasmic compartment, which facilitates correct bond formation between the HC and LC of Fab fragments. While *E. coli* has many advantages including high cell density and low media cost, protein production with this host system may result in the formation of misfolded proteins within inclusion bodies that require a lengthy in vitro refolding process.

Unfortunately, the yields from Fab expression in *E. coli* have remained low due to intracellular degradation, inefficient translocation and degradation (Ellis et al. 2017). Together, this results in difficulties in scaling-up and purifying these proteins leading to increased costs, one of the key advantages of this expression system. Recently, Fab production in other expression systems have seen improvements in yield. Fab expression in yeast has been reported at up to 50 mg/L in shake flasks (Ellis et al. 2017). While cell free systems still lag behind cell-based expression systems, expression of soluble Fab has been reported at 1mg from 1ml of solution (Matsuda et al. 2018).

It is important to note that during the antibody development process an unpaired cysteine may be chemically reactive and lead to heterogeneity of the product and variations of affinity (Gadgil et al. 2006, Eon-Duval et al. 2012). It can also promote misfolding or aggregation due to pairing with other cysteines in the variable region. The unpaired cysteine may also lead to reduced yields. Further to this, the thioether exchange with metabolites and serum proteins

may reduce conjugate stability and in turn decrease *in vivo* efficacy (Patterson et al. 2014). Classically, maleimide conjugation is a heterobifunctional crosslinker and it is often difficult to achieve a 1:1 protein: conjugate ratio. However, during the design only one cysteine was included to maximise our antibody homogeneity. While the unpaired cysteine was employed in this iteration of the humanised Fab, recent advances in bioconjugation may be preferable. For example, selenocysteine incorporation and conjugation with allenamides, iodacetamide or sulfone compounds has been demonstrated to produce stable homogenous antibody-drug conjugates (Li et al. 2015, Hofer et al. 2008, Li et al. 2014). However this technique may not be readily transferable as it requires intensive screening procedures to identify suitable positions for modification. Forte et al. (2018) demonstrated reduced hydrolysis and higher reactivity with cysteines using next generation maleimides such as diiodomaleimide.

Interestingly, LO1 was a simple antibody to humanise as it had no structural or sequence-based predicted modification sites. Further to this, many positions identified as important for the humanisation of murine antibodies were already present on the acceptor framework. For example, Zhang & Ho (2016) reported the proline in VH, position 41 to be important to retain during humanisation. This was already present in the murine sequence and on the acceptor framework so mutations were not necessary here.

Despite the lack of structural analysis or deep mutagenesis data, the humanisation of LO1-huFab improved our understanding of which residues may be important for antigen binding. It is now known that the residue on VH, position 91 is important in the humanised forms as with this backmutated expression of the Fab was abrogated. Furthermore it is now known that three mutations in positions 48, 78 and 69 significantly elevated the immunogenic profile. Alongside our prior information of the two lysines in CDR-H3 being important for antigen binding, this brings us one step closer to understanding the molecular basis of H1L0 antigen binding.

Chapter 5

In vivo imaging of an oxLDL humanised Fab in a murine model of atherosclerosis

5.1 Introduction

Molecular imaging is based on the principle that pathological changes occur at the cellular level in advance of anatomic changes in the diseased area. This can allow for the early detection of disease when anatomical changes may be minute. NIRF imaging provides superior photon penetration to visible light and is minimally affected by autofluorescence from haemoglobin (Smith et al. 2009). Advances in imaging modalities have led to the introduction of FMT which can obtain high sensitivity detection while utilising readily available dyes and probes. In this chapter, I continue the work on the humanised Fab from Chapter 4. H1L0 was conjugated to a near-infrared fluorophore for *ex vivo* validation using mouse atherosclerotic tissue sections and subsequently validated *in vivo* using FMT. CT imaging was performed for anatomical co-registration with the molecular data.

5.2 Results and Discussion

5.2.1 Immunohistochemistry of mouse aortic root

Without the LDLR, macrophages in LDLR^{-/-} mice continually endocytose oxLDL through their scavenger receptors. These receptors lack the negative feedback loop that governs the physiological LDLR. Thus, a high fat diet (HFD) supplemented with cholesterol will lead to the induction of atherosclerotic lesions in LDLR^{-/-} mice (Teupser et al. 2003). After a prolonged period on HFD the mice develop lesions on the aortic root as demonstrated by a thickening of the arterial walls and valves (Figure 5.1A). H1L0 (huFab) was conjugated with VivoTag-S-750-MAL to generate huFab-750. Immunohistochemistry using huFab-750 demonstrated focal staining within the core of plaques on both the arterial wall (Figure 5.1B) and the valves (Figure 5.1D). The signal from huFab-750 did not colocalise with that of 4',6-diamidino-2-phenylindole (DAPI) (nuclear stain, blue) or with the green autofluorescence from the extracellular matrix (Figure 5.1B, D). Focal localisation was not present when staining serial sections with a control human Fab (Figure 5.1C, E), demonstrating specificity of the huFab-750 staining. The staining of huFab-750 was predominantly extracellular, in areas void of signal from the ECM. This suggests the Fab is staining MDA-LDL in areas which are void of cells or apoptotic cells, such as the necrotic core. Similar to the full mAb (Khamis et al. 2016), this pattern of staining may be due to apoptotic cells releasing their intracellular matter including oxLDL, suggesting a failure in the clearance of macrophage, which is a feature of advanced atherosclerotic lesions (Virmani et al. 2006). This failure of apoptotic cell uptake ("efferocytosis") is likely to aggravate the inflammation and lead to unstable, necrotic plaques which are rupture-prone. Schrijvers et al. (2005) identified that defective efferocytosis was 19 times higher in atherosclerotic lesions than other human tissue. Recent studies have identified genes and pathways which become dysregulated in patients with defective efferocytosis (Yin 2019).

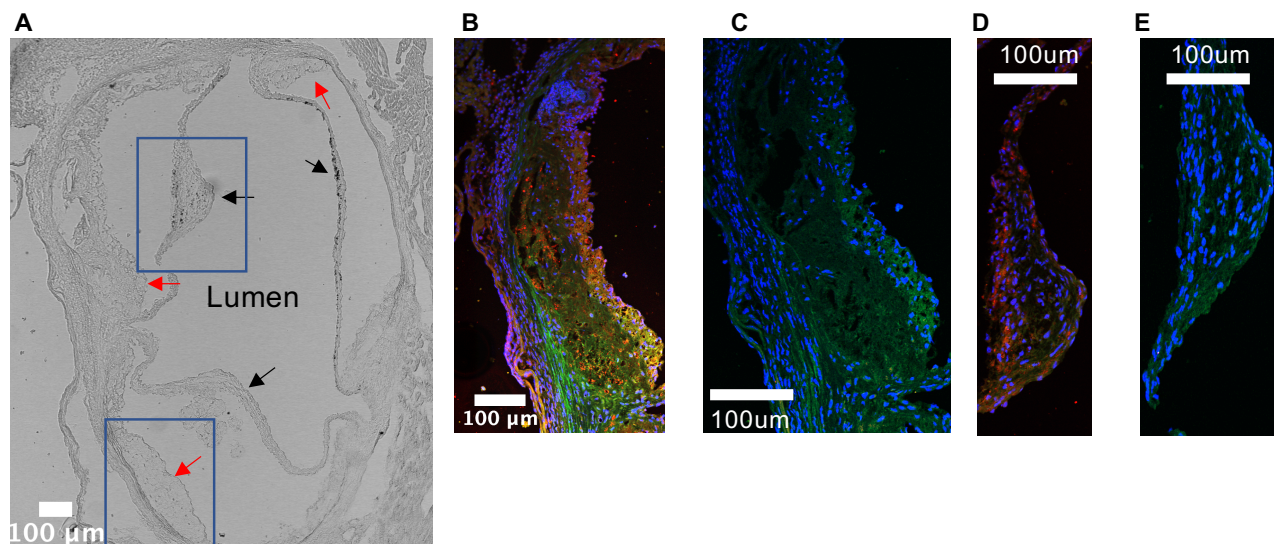


Figure 5.1: Immunohistochemistry of huFab-750 and control staining mouse aortic root. A) Brightfield image of the aortic root of a LDLR^{-/-} fed a high fat diet for 22 weeks. Black arrows indicate the three valve leaflets. Red arrows indicate thickening of the arterial wall. Boxes are regions of interest analysed further subfigures. B) Aortic root stained with huFab conjugated with a NIR dye. HuFab-750 signal (red) is located in the necrotic core, C) Serial section of the aortic root stained with a human Fab control conjugated with NIR dye. Red = HuFab-750 (B,D) or control-huFab-750 (C,E), green = autofluorescence, blue = 4',6-diamidino-2-phenylindole (DAPI).

5.2.2 *In vivo* studies of huFab

To induce atherosclerosis in our mice, the experimental protocol for the *in vivo* studies was such that once female LDLR^{-/-} mice reached 10 weeks of age, they were transitioned onto a HFD for 30 more weeks. They were then injected with the respective probes and imaged using FMT-CT (Figure 5.2A). The animals increased in weight as they aged (Figure 5.2B), with no significant difference in weight between the LDLR^{-/-} and wild-type (WT) mice (Figure 5.2D).

Blood clearance of huFab-750

Half-life determination is an important step in determining optimal imaging time, as it can be used to minimise target-to-background ratio. The half-life of Fab fragments in mice has been reported to range from a few minutes to 84 hours (Mozdzanowska et al. 2003, Adams et al. 2016). In comparison, the circulation half-life of a full mAb is estimated to be 2-3 days in mice (Chapman et al. 1999). The parent LO1 IgG closely matched this with an estimated half-life

of 21-hours (Khamis et al. 2016). Unfortunately, a key hindrance during our LO1 whole IgG studies was the high levels of signal to noise background due to its large size (150kDa) and the presence of the FcRn receptor resulting in antibody recycling. It was proposed that a humanised Fab fragment (ie. H1L0 huFab), which is smaller (48kDa) and lacks the FcRn receptor would retain the specificity and antigen-binding of the parent antibody while demonstrating improved pharmacokinetics for tissue penetration and imaging.

One nanomole of hu-Fab-750 was injected, after which plasma fluorescence was measured over 30 hours using serial tail-bleeds. It was hypothesised that due to the Fab's small size (48kDa), it would undergo rapid drug elimination through glomerular filtration within the kidneys. The blood clearance analysis validated this hypothesis as a one-phase decay estimates a 12-minute half-life for the Fab (Figure 5.2D). This was further corroborated by the high fluorescence signal in the kidneys in Figure 5.8A due to the accumulation of the fluorescently labelled Fab. An imaging time point of four hours was chosen, as this was the time equivalent to the imaging time point with the full mAb and would allow 16 half-lives to have passed prior to imaging, reducing the likelihood of high background signal.

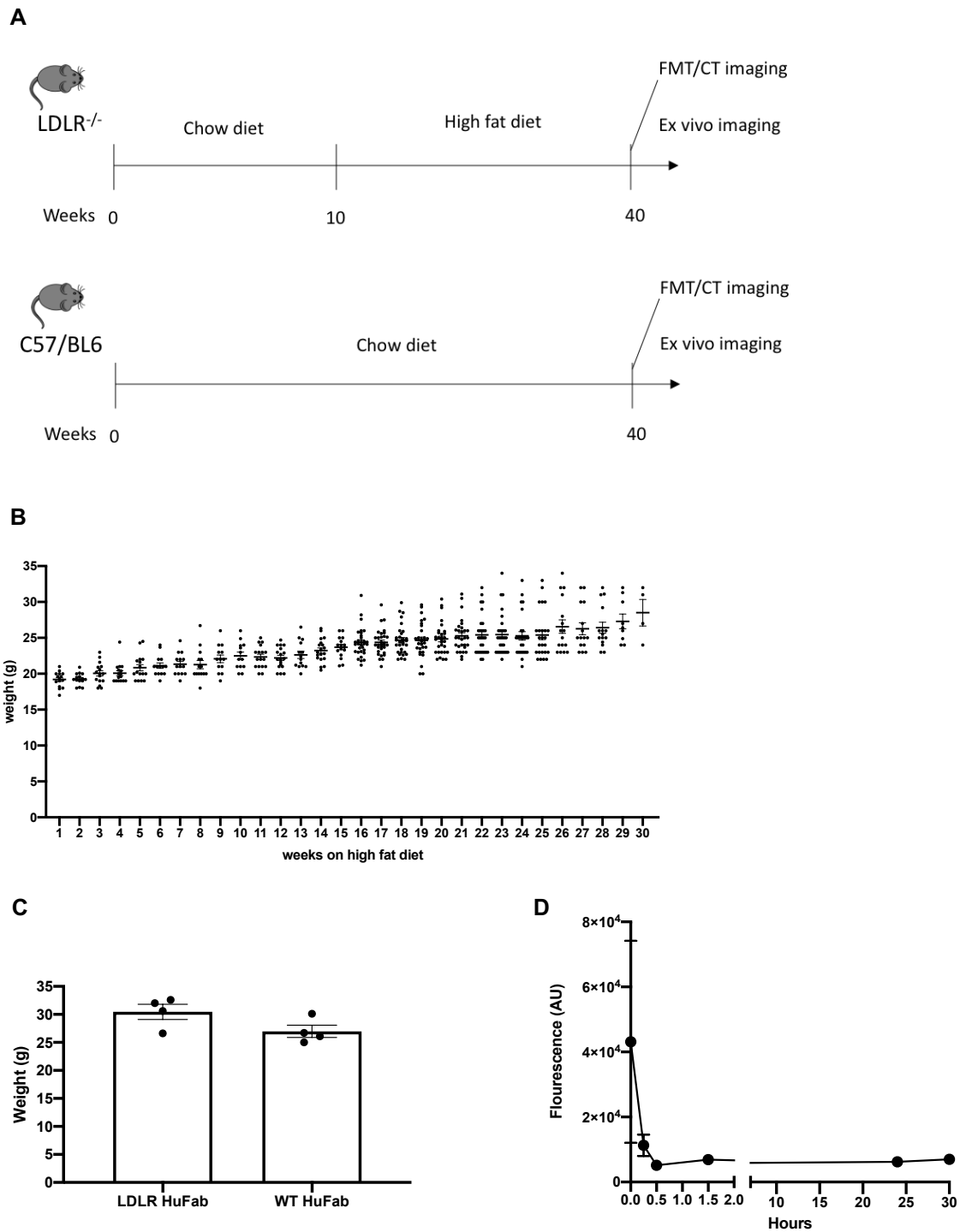


Figure 5.2: Characteristics of the mouse colony. A) Experimental set up and feeding schedule for $LDLR^{-/-}$ and C57/BL6 mice. B) Weight of $LDLR^{-/-}$ mice once they are on a high fat diet. Values are mean $\pm$ SEM. C) Weights of the $LDLR^{-/-}$ mice and C57/BL6 mice used for *in vivo* studies, $p = 0.09$, ns, unpaired T-test with Welch's correction. D) The clearance of LO1-huFab-750 from the blood of C57/BL6 mice. Values are mean $\pm$ SEM.

***In vivo* imaging of huFab-750**

Fluorescence imaging provides a robust and sensitive method for observing biological processes. Although, proteins have intrinsic fluorescence from their aromatic residues, extrinsic labels provide advantages when observing biological processes such as the ability to choose the label itself and its location. The Fab fragment has four cysteine residues which are involved in forming disulphide bonds between the HC and LC. The previous studies with the full antibody led to the understanding that lysine residues on LO1 were crucial for binding to antigen. Therefore, a cysteine residue was introduced through the inclusion of the natural IgG1 upper hinge region as described in Chapter 3 for site-specific conjugation of fluorophore. As this residue is engineered into the C-terminus it is unlikely to interfere with epitope binding, either on its own or once conjugated with the fluorophore.

FMT imaging provides a means for quantifying NIRF signal as it is capable of acquiring three-dimensional signal and reconstructing it non-invasively. The modality currently allows multiplexing with up to four probes as the device is equipped with four different lasers (635, 680, 750, 800nm). Three probes with different emission and excitation wavelengths were delivered to each mouse to maximise the data generated. These were 1) huFab (1nmol) conjugated with VivoTag-750-MAL (huFab-750) to identify the presence of MDA-LDL within the mouse; 2) MMPSENSE 645 FAST (MMPsense) (2nmol). MMPsense is cleaved in the presence of within generating a fluorescence signal, allowing the identification of areas of inflammation and colocalisation analyses to identify regions with both high oxLDL and inflammation levels; 3) IntegriSense 680 (IntegriSense) (4nmol) to identify $\alpha v \beta 3$ integrins which are known to be upregulated in sites of angiogenesis. All three probes were co-administered intravenously and the mice imaged four hours later based on the blood clearance of all three probes according to our data and manufacturer's recommendation (Figure 5.2C). Contrast-enhanced CT was performed for anatomical coregistration using Omnipaque-300 using a dose of 1.5 g of iodine/kg (3.23 g iohexol/kg).

FMT-CT coregistration demonstrated that LO1-huFab localised to focal areas of the as-

ascending thoracic aortic and aortic arch in LDLR^{-/-} (Figure 5.3, 5.4Ai-iii) and this was not observed in WT mice (Figure 5.5Ai-iii). As shown in Figure 5.6A this targeting was validated by quantification of the huFab signal which showed a significant difference between LDLR^{-/-} and WT mice (63.16 ± 12.64 pmol vs 23.92 ± 4.35 pmol, $p=0.045$, $n=4$ per group). MMPsense (35.40 ± 7.12 pmol vs 20.44 ± 1.87 pmol, $p=0.012$, $n=4$ per group) and IntegriSense (274 ± 66.20 pmol vs 96.03 ± 3.139 pmol, $p=0.074$, $n=4$ per group) were not statistically significantly different between LDLR^{-/-} and WT mice (Figure 5.6). However, this may be due to small group size, as both of these probes may reach statistical significance with larger numbers. Excitingly, however, the results suggest that it is possible to sensitively detect changes in MDA-LDL prior to the increased levels of inflammation and angiogenesis present in atherosclerotic plaques. This is consistent with the theory that an accumulation of MDA-LDL drives further inflammation and angiogenesis. Coregistration of biological data with anatomical data revealed that LO1-huFab, IntegriSense and MMPsense localised distinctly from one another. Figure 5.6A demonstrates LO1-huFab targeting the aorta running right alongside the spine. In contrast, both IntegriSense and MMPsense targeted within the heart and minimally in the aorta itself. Furthermore, localisation of LO1-huFab-750 to lesional areas was confirmed *ex vivo* with fluorescence reflectance imaging (FRI) (Figure 5.7).

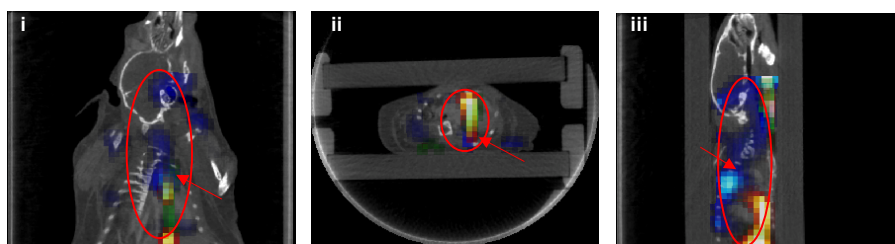


Figure 5.3: Representative images of FMT-CT colocalisation of huFab, IntegriSense and MMPsense. huFab (blue), IntegriSense (green) and MMPsense (red) signals overlaid in a diseased LDLR^{-/-} mouse fed a high fat diet for 30 weeks. Red circles indicate the region of interest utilised for the quantification of signal in Figure 5.6. Red arrows indicate the aorta. Panels arranged in coronal, axial and sagittal slices (left, centre, right, respectively).

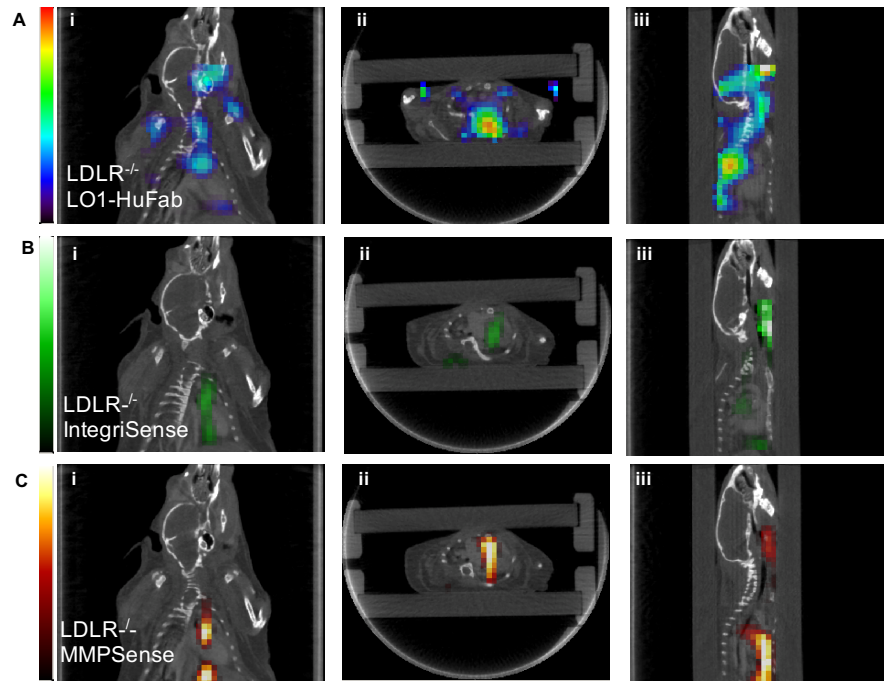


Figure 5.4: Representative images of FMT-CT colocalisation of huFab, IntegriSense and MMP5ense in a diseased $LDLR^{-/-}$ mouse. huFab (A), IntegriSense (B) and MMP5ense (C) signals in a $LDLR^{-/-}$ mouse fed a high fat diet for 30 weeks. $N=4$ for all groups. Panels arranged in coronal (i), axial (ii) and sagittal (iii) slices (left, centre, right, respectively.)

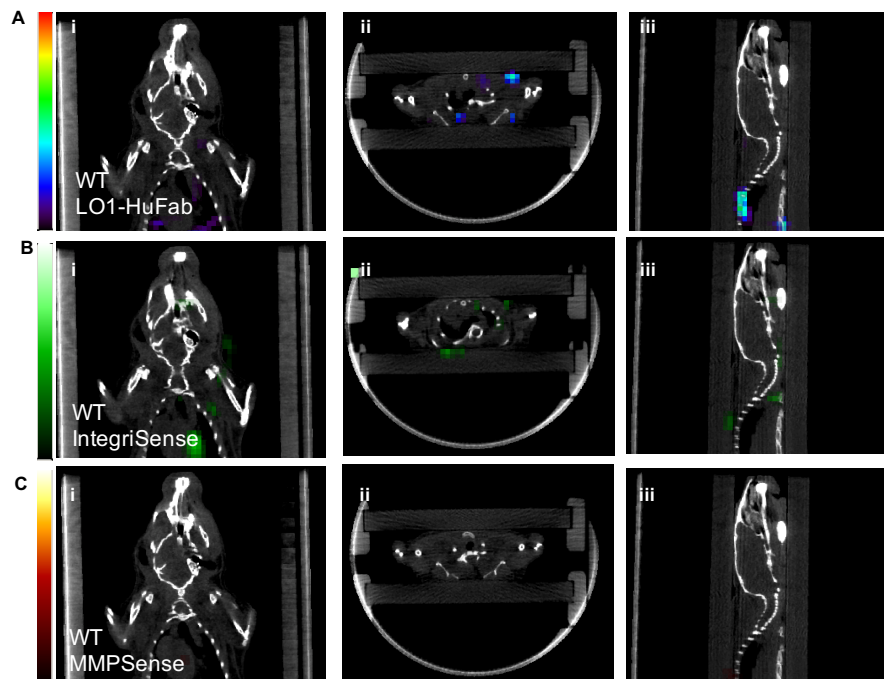


Figure 5.5: Representative images of FMT-CT colocalisation of huFab, IntegriSense and MMP5ense in a C57/BL6 (WT) mouse. huFab (A), IntegriSense (B) and MMP5ense (C) signals in a C57/BL6 (WT) mouse on a chow diet. $N=4$ for all groups. Panels arranged in coronal (i), axial (ii) and sagittal (iii) slices (left, centre, right, respectively.)

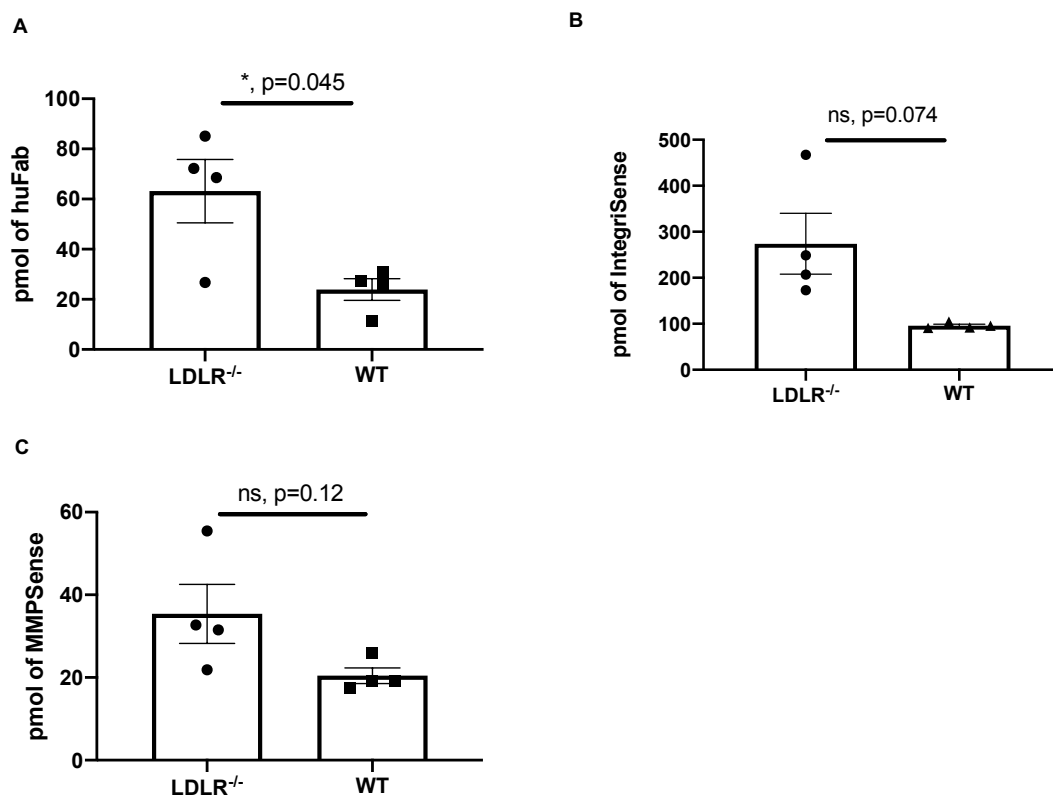


Figure 5.6: Quantification of FMT signal of LO1-huFab, IntegriSense and MMPSense Signal of A) LO1-huFab B) IntegriSense C) MMPSense in LDLR^{-/-} mouse fed a high fat diet for 30 weeks and age-matched C57/BL6 (WT) mice. Quantification performed in the region of interest identified in Figure 5.3. N=4 for all groups. Values are mean ± SEM of biological replicates. Statistical significance calculated using unpaired T-test with Welch's correction.

5.2.3 *Ex vivo* imaging of aorta

Aortas were extracted from animals injected with LO1-huFab-750, IntegriSense-680 and MMPSense-645 and fluorescence reflectance imaging was performed. The extracted aortas were washed and immediately imaged *en face*. It is clear in Figure 5.7A,B that lesions can be seen in the aortic arch in LDLR^{-/-} mice which are largely absent from the WT mice. These lesions are observed in the inner curvature of the aorta as well as in the abdominal aorta. FRI demonstrated that all three probes were detected in LDLR mice, but not in WT mice with the exception of MMPSense (Figure 5.7Ci-vi). HuFab and IntegriSense signal were seen *ex vivo* in regions correlating to the aortic root in LDLR^{-/-} mice. LO1-huFab signal displayed focal signal in the aortic root and abdominal aorta (Figure 5.7Ci). The heavily diseased aortic arch

displayed higher levels of signal compared to the smaller plaques in the abdominal aorta. This difference in signal intensity suggests that LO1-huFab is able to distinguish different levels of disease. In contrast, IntegriSense signal was observed throughout the aorta, with high signal in the aortic arch and the abdominal aorta regions (Figure 5.7Cii). MMPsense signal was concentrated in the aortic root. Surprisingly, MMPsense signal was observed throughout the aorta of WT mice as well. This may be due to the age of the mice. Similar to humans, increased levels of inflammation are observed in mice as they age (Pettan-Brewer & M. Treuting 2011, Ray et al. 2010).

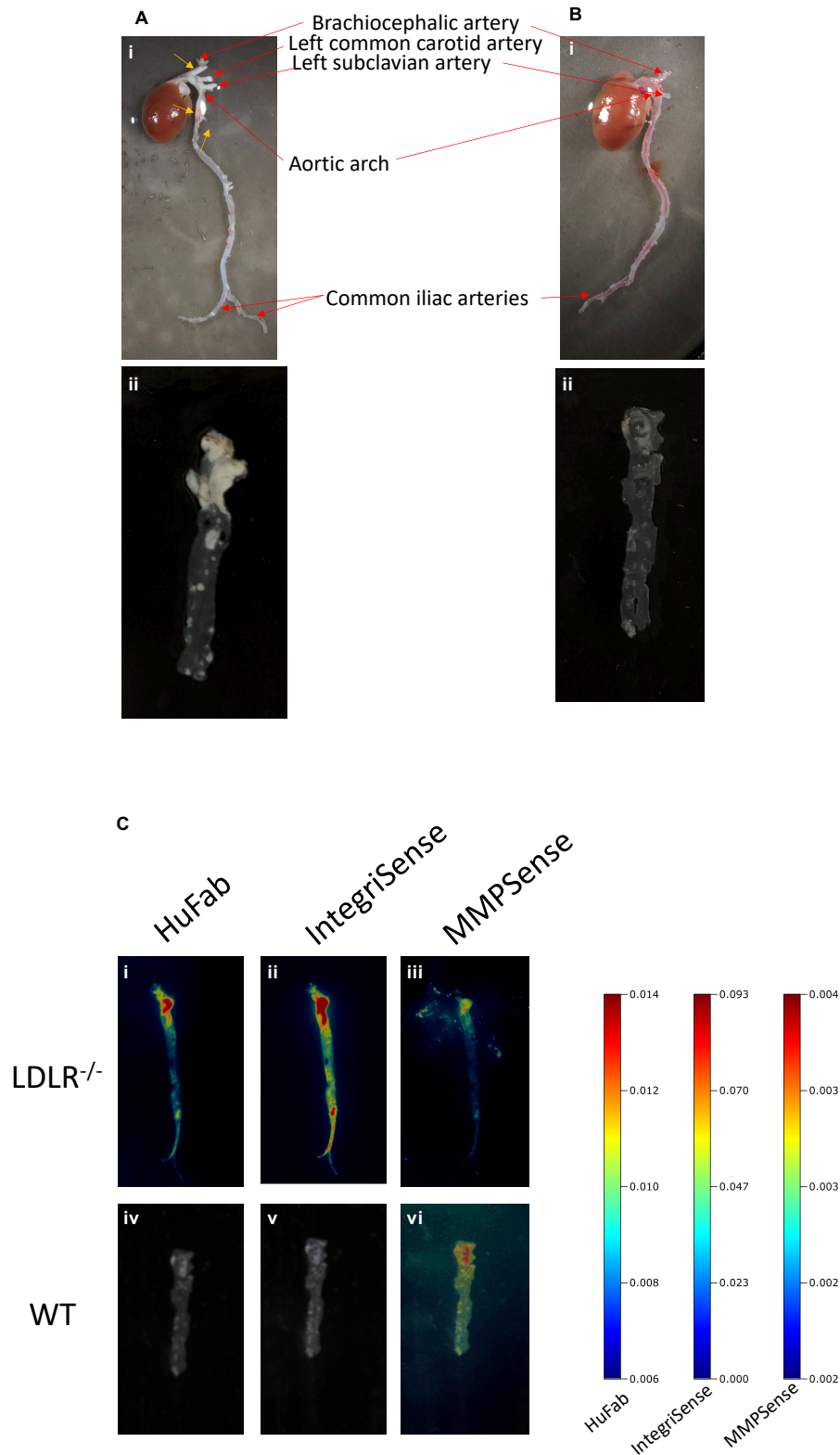


Figure 5.7: *Ex vivo* fluorescence reflectance imaging. A) White light images of a heart and aorta extracted from a $LDLR^{-/-}$ mouse and B) from a C57/BL6 mouse at 40 weeks of age. Yellow arrows indicate thickening of the vessel wall. C) Fluorescence reflectance imaging of aortas extracted from $LDLR^{-/-}$ and C57/BL6 mice after injection with LO1-huFab-750, IntegriSense-680 and MMPsense-645. N=4 for all groups.

Organ distribution of huFab, IntegriSense and MMPSense

The three imaging probes were injected and animals imaged four hours later on the FMT. Organs were immediately harvested and imaged to obtain the distribution of huFab, IntegriSense and MMPSense in WT and LDLR^{-/-} mice. As expected the majority of huFab localised to the kidneys in both WT and LDLR^{-/-} mice, and this is explained by its molecular weight being below the renal cut-off. This was in contrast to our finding with the full mAb LO1 which predominately was identified in the liver (Khamis et al. 2016). Both of these results are consistent with theory and research on the pharmacokinetics of protein molecules of the respective sizes.

Interestingly, a difference was observed in the liver quantities of huFab in WT and LDLR^{-/-} mice (145.23 ± 13.90 pmol vs 101.72 ± 4.14 pmol, $p=0.044$, $n=4$ for both groups), which may indicate liver-specific targeting of oxLDL by huFab in LDLR^{-/-} mice (Figure 5.8A). Alternatively, this signal may be occurring from huFab bound to plasma oxLDL being transported to the liver for processing. Supporting both of these hypotheses, Van Berkel et al. (1991) demonstrated that presence of oxLDL within the liver as it is rapidly taken up *in vivo* by Kupffer cells. IntegriSense was elevated in the liver (613.03 ± 63.44 pmol vs 164.62 ± 18.48 pmol, $p=0.0003$, $n=4$ for both groups) and kidneys (103.09 ± 6.34 pmol vs 66.53 ± 5.5 pmol, $p=0.011$, $n=4$ for both groups) in LDLR^{-/-} mice as compared to WT mice (Figure 5.8B). MMPSense was significantly elevated in all three organs in the LDLR^{-/-} mice as compared to WT mice (Figure 5.8C). Together, these results indicate high levels of inflammation and angiogenesis occurring within the liver, kidney and spleen.

In humans, the risk factors for atherosclerosis and conditions that cause nonalcoholic steatohepatitis (NASH) are very similar and the conditions may often present together. NASH is characterised by inflammation, oxidative stress, hepatic injury and fibrosis (Lytle & Jump 2016). While further validation in relevant models needs to be conducted, these results may indicate that huFab, IntegriSense and MMPSense and the use of NIRF could be used to detect conditions such as NASH.

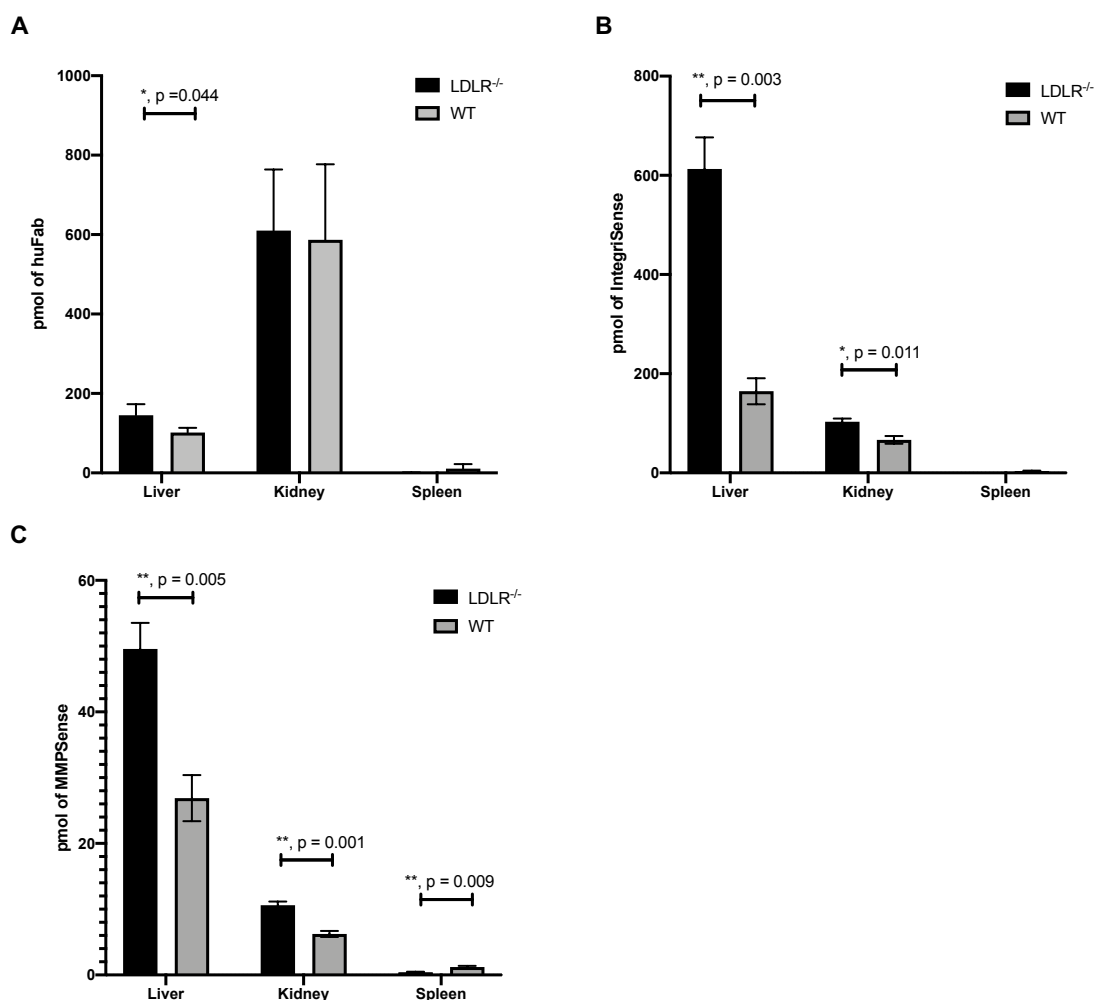


Figure 5.8: Organ distribution of huFab, IntegriSense and MMPSense. A) The organ distribution of huFab-750 in primary metabolic organs. B) The organ distribution of IntegriSense-680 in primary metabolic organs. C) B) The organ distribution of MMPSense in primary metabolic organs. Values are mean $\pm$ SEM of biological replicates. Statistical significance calculated using unpaired T-test with Welch's correction. N=4 for all groups.

5.2.4 Whole mouse signal quantification of huFab, IntegriSense and MMPSense

It has been generally accepted that highly oxidised LDL is not maintained in the circulation due to the high levels of antioxidants in blood. Furthermore, these highly oxidised LDL particles would be expected to be rapidly cleared by scavenger receptors within the liver (Van Berkel et al. 1991). The oxLDL found in blood is generally bound by proteins complexes trafficking it to be processed (Lopes-Virella & Virella 2010). Therefore, while the measure of oxLDL has been

correlated to coronary artery disease, the lack of a direct relationship between plasma oxLDL and adverse clinical events may be due to measuring only a tiny proportion of the oxLDL load within a patient.

A measure of whole body oxLDL load may be beneficial in determining patients with specifically higher oxLDL loads, in line with the concept of identifying the “vulnerable patient”. A vulnerable patient may be defined as one with high atherosclerotic burden, vulnerable plaques or thrombogenic blood. A pilot study investigated whether a diseased animal (LDLR^{-/-} mouse) could be distinguished from a healthy animal (WT mouse) based on whole body oxLDL signal. This demonstrated that of the three probes injected only the humanised Fab (huFab) was able to detect a difference between diseased and non-diseased animal (Figure 5.9A, 1382 ± 159.0 pmol vs 725.5 ± 121.6 pmol, $p = 0.018$, $n = 4$ for both groups). The analysis was normalised to the weight of each mouse to investigate whether this was weight dependent. This demonstrated a slight negative correlation with weight ($R^2 = 0.5262$ for LDLR^{-/-}, $R^2 = 0.3154$ for WT, data not shown) and a statistically significant difference was not observed between the groups when normalised by weight (Figure 5.9B, 46.23 ± 7.33 pmol vs 27.34 ± 5.43 pmol, $p = 0.08$). It is plausible that this is due to the group size and an increased ‘n’ number would gain significance even when normalised by weight. Figures 5.9C-F demonstrate that both IntegriSense and MMPSense were not significantly different between LDLR^{-/-} and WT groups with or without normalisation to weight demonstrating that these probes alone were not able to distinguish diseased animals from non-diseased ones. The finding that only the huFab was able to distinguish diseased and non-diseased animals on a whole-body level is promising. Apart from identifying the vulnerable patient, huFab could also be used to monitor the effect of treatments on oxLDL levels.

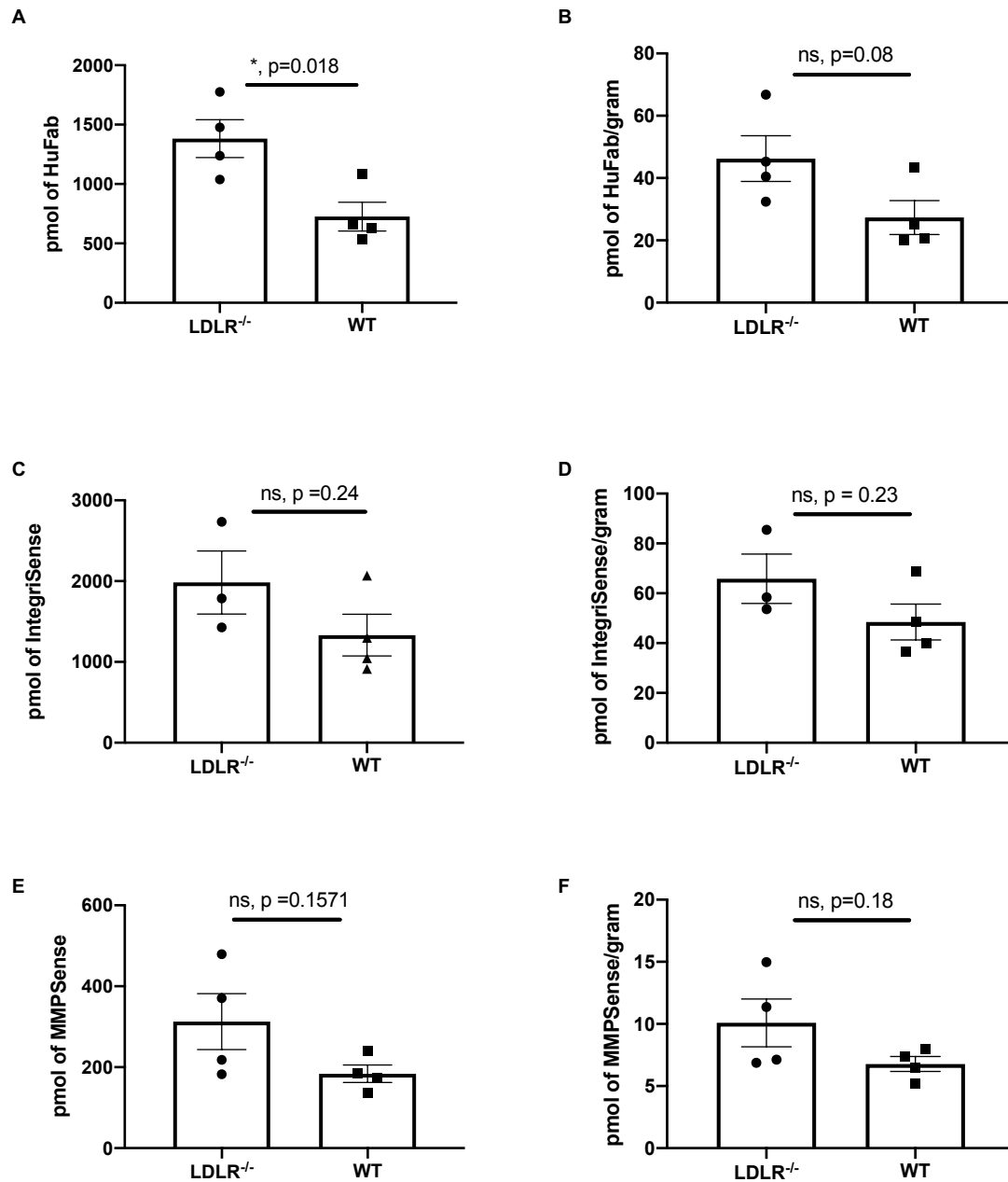


Figure 5.9: Whole body quantification of huFab, IntegriSense and MMPSense. A) Quantification of huFab in the whole mouse of LDLR^{-/-} and WT and B) normalised by weight of the mouse. C) Quantification of IntegriSense in the whole mouse of LDLR^{-/-} and WT and D) normalised by weight of the mouse. E) Quantification of MMPSense in the whole mouse of LDLR^{-/-} and WT and F) normalised by weight of the mouse. Values are mean $\pm$ SEM of biological replicates. Statistical significance calculated using unpaired T-test with Welch's correction. N=4 for all groups.

5.3 Conclusion

A multitude of factors contribute to the development of atherosclerotic plaques and their vulnerability. This poses a significant challenge as it is difficult to determine and monitor a sole attribute of plaque vulnerability. However, this also provides a unique opportunity with emerging technologies such as NIRF imaging. In this chapter, I have utilised the multiple factors as an advantage to aid in colocalisation of atherosclerotic signal and to identify that changes in MDA-LDL appear to precede that of matrix metalloproteinases (inflammation) and integrin $\alpha v \beta 3$ (angiogenesis).

This chapter builds on the *in vitro* generation and characterisation of huFab described in Chapter 3 through the characterisation within a preclinical atherosclerosis model. The first section of this chapter begins with the *ex vivo* validation of huFab binding to oxLDL within mouse atherosclerotic lesions. The latter section of this chapter is focused on the *in vivo* targeting and quantification of the three different probes within different regions of the body. The results demonstrated that huFab localised to MDA-LDL within the LDLR^{-/-} mouse model and targeted the heavily diseased aortic arch and its bifurcations. HuFab signal was also identified in plaques within the abdominal aorta with weaker signal. The studies were further able to distinguish diseased and non-diseased animals from one another both within the aortic arch region and on a whole body basis demonstrating that it could be more widely used to identify the vulnerable patient. While three probes were injected for the *in vivo* studies, it was found that only huFab detected a significant difference between WT and LDLR^{-/-} animals in agreement with our current understanding of atherosclerosis pathobiology that the buildup of lipid precedes the subsequent inflammation and angiogenesis. This suggests that until the main cause of a build-up of oxLDL is addressed, treatments to ameliorate inflammation or angiogenesis may only bring limited benefit when it comes to clinical adverse events.

Chapter 6

Single-chain Variable Fragment generation from full mAb

6.1 Introduction

It is well established that Fv fragments have limited *in vivo* utility due to the dissociation of the VH and VL from one another. This has been overcome by genetically linking the two variable fragments to generate scFvs. These variable domains subsequently have the flexibility and space to fold and interact in a similar manner to mAb Fv domain. Due to its small size the scFv is predicted to have better penetration within atherosclerotic lesions than other antibody fragments of LO1. However, due to its monovalency, it may suffer from a reduced avidity effect and may not be retained within the plaque as effectively. Thus, it is hoped that a diabody can also be generated to investigate the impact of avidity on fragment retention. A diabody is similar in size to a Fab fragment, but contains two ABS, making it bivalent.

6.2 Results and Discussion

6.2.1 Design of scFv and diabody fragments of LO1

The scFv was generated from the murine LO1 sequence. Due to the scFvs being of murine origin, detection of these fragments for downstream purification and characterisation required the incorporation of an affinity tag. A polyhistidine tag was chosen to allow downstream processes such as affinity purification and ELISA to be carried out using a singular tag. Due to small size of the polyhistidine tag (2.5 kDa) it is not thought to interact with the function of a scFv. For *in vivo* studies, the removal of the polyhistidine tag may be required, and therefore the tobacco etch virus (TEV) motif was introduced. The TEV protease specifically cleaves the ENLYFQG consensus sequence, thereby removing the polyhistidine tag. Furthermore, there is a growing understanding that the assumption that a polyhistidine tagged protein and its equivalent untagged protein may not behave the same (Booth et al. 2018, Majorek et al. 2014). Although these studies have been overwhelmingly performed on enzymes. A polyhistidine tag has also been shown to improve solubility of proteins due to its positive charge (Hammarström et al. 2009). Whether the polyhistidine tag is better placed on the N or C-terminal has to be determined empirically.

Direct fusion of the VH and VL domains may result in unfavourable outcomes including low yield, misfolding or low/non-functional protein (Zhao et al. 2008, Amet et al. 2009). Generally, effort is focused on the protein domains to be fused, while the linker choice is overlooked. Therefore, an approach of rational design was implemented for linker choice in the LO1-scFv. Glycine serine linkers have become a mainstay in protein fusions due to their flexibility, solubility and high stability *in vivo*. It consists of a sequence of four glycine residues followed by one serine residue where the serine residues are hydrophilic residues and allow the protein to form hydrogen bonds with the buffer and the glycine residues provide the linker with flexibility to adopt a range of conformations (Huston et al. 1988). The hydrophilic serine residues are additionally unlikely to interact with the hydrophobic patches that generate the VH-VL interface. The

glycine and serine motif is repeated an ‘n’ number of times, with the repetitive units ranging from 1-5, known as the $(G_4S)_n$ linker. Studies have demonstrated that longer linkers generally favour the formation of scFv and shorter linkers favour the formation of diabodies; with the optimal length of the $(G_4S)_n$ linker for scFv and diabody formation determined empirically for each mAb. In this thesis, five different linkers were trialled for linking LO1 VH and VL sequences. Four of them were the $(G_4S)_n$ linker, ranging from one to four repeats. The final linker was designed by Whitlow et al. (1993) and is known as the ‘218’ linker. The sequence has the underlying glycine and serine residues with the addition of three charged residues to enhance solubility. Furthermore, studies with this longer 18-mer linker demonstrated increased affinity compared to shorter linkers. Yusakul et al. (2016) demonstrated that a scFv with a longer $(G_4S)_n$ linker displayed higher inhibitory activity than those with shorter ones. Apart from the linker, the scFv were designed to all contain identical VH and VL sequences. The five different linker sequences are shown in Table 6.1.

The terminology for the scFv is as follows: when the scFv is in the orientation VH-VL orientation, the first number is a 1 and when in the VL-VH orientation the first number will be a 2. This first number will be followed by a number ranging from 1-5 related to their linker. For example, construct 1.2 will be in the VH-VL orientation with a $(G_4S)_2$ linker.

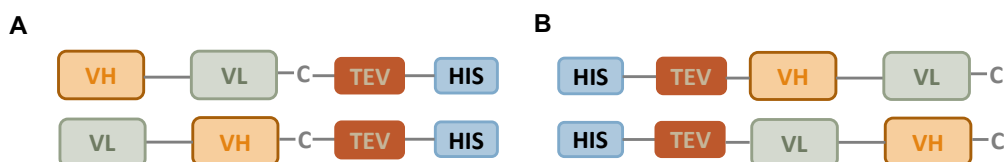


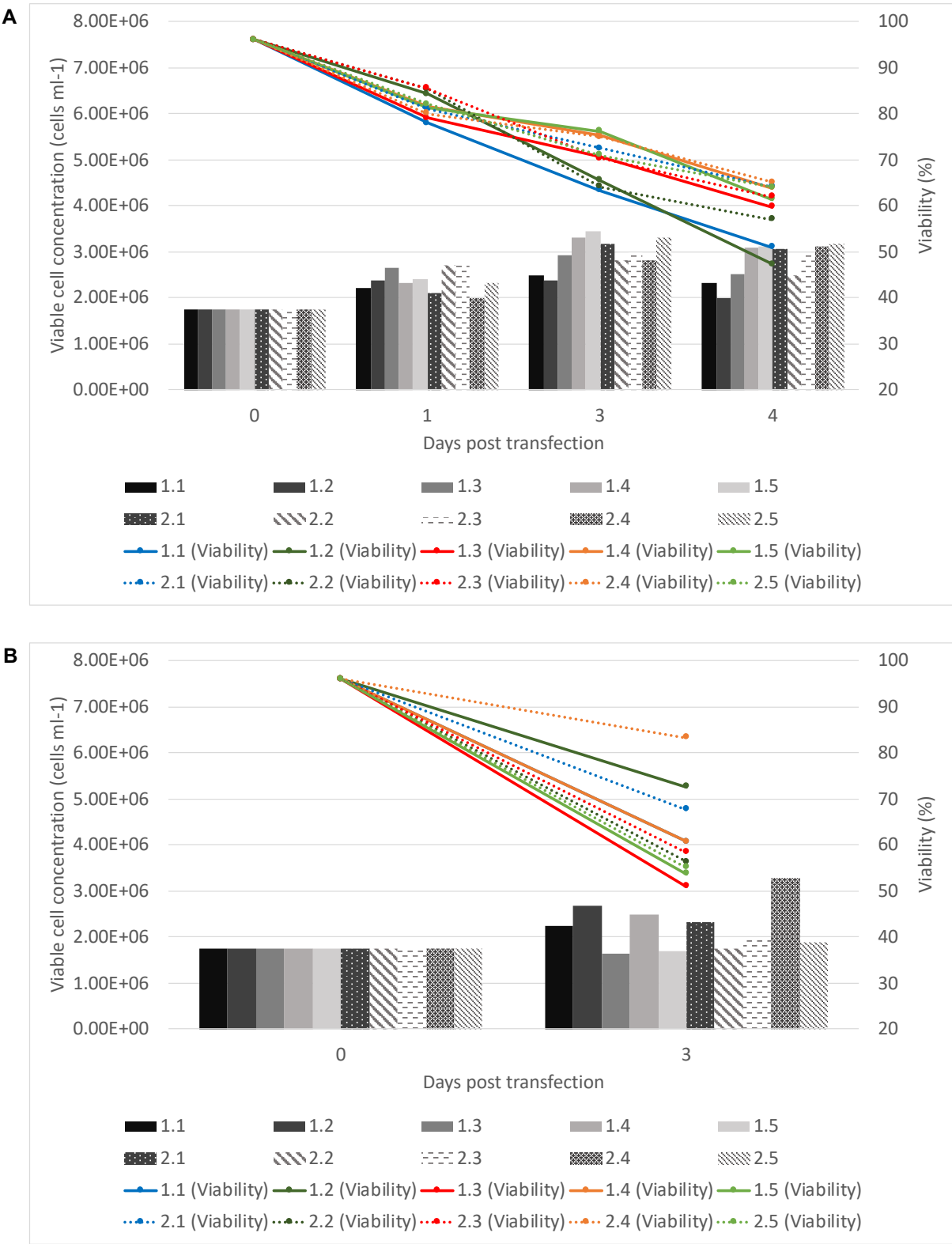
Figure 6.1: Design of LO1 scFv constructs. Polyhistidine tag with tobacco etch virus motif was tested in both the C (A) and N (B) terminus of the scFv. VH-VL and VL-VH orientations were tested. An extra cysteine residue was incorporated for conjugation to fluorophore. Five different linkers were tested for joining the VH and VL domains.

Table 6.1: Linkers incorporated into LO1 scFv design. First number in the variant identifier relates to the orientation while the second number relates to the linker.

Name in thesis	Shorthand name	Sequence	Number of residues	Citation
.1	(G ₄ S) ₁	GGGGS	5	Huston 1988
.2	(G ₄ S) ₂	GGGGSGGGGS	10	Huston 1988
.3	(G ₄ S) ₃	GGGGSGGGSGGGGS	15	Huston 1988
.4	(G ₄ S) ₄	GGGGSGGGSGGGSGGGSGGGGS	20	Huston 1988
.5	218	GSTSGSGKPGSGEGSTKG	18	Whitlow 1993

6.2.2 Viability analysis of mammalian cells expressing LO1 scFv

As shown in Figure 6.1, LO1 scFv variants were constructed with either a C or N-terminal extension of a polyhistidine tag and expressed in HEK293 cells at a small scale of 20 mL. The five different linkers and two different orientations generated a panel of 20 different constructs. These constructs were to be expressed only until their viability as a group reached 80% or below. Following transfection, the cell culture was sampled in order to investigate the culture viability and viable cell concentration. However, after only one day, transfected cells had reached the threshold of falling below 80%. In the hope that this reduction in viability was due to the stress of transfection and the cells would recover, the threshold was lowered to 60% at which point the productivity and cell debris would impact expressed protein quality (De Los Milagros Bassani Molinas et al. 2014). Cells transfected with the N-terminal polyhistidine tag reached a threshold of 60% of lower viability on day three post-transfection, while C-terminal polyhistidine tagged constructs reached this threshold on day four (Figure 6.2A,B). This rapid decline in viability was unexpected and surprising as it is common to express mAbs for several days in HEK293 cells. Therefore, to continue the expression in mammalian cells, the Expi293 cell system was explored. This expression system utilises a medium that allows cells to grow to higher densities with high viability (Jones et al. 2012). This, in turn, can increase the protein production capacity per millilitre of culture media.



As expected, when the viable cell concentration and culture viability were investigated post-transfection they were found to be higher with Expi293 cells seen in Figure 6.3 than with HEK293 cells. However, the faster decline in both variables was again observed with the N-terminal polyhistidine tagged proteins. The supernatants were analysed by SDS-PAGE to determine if protein expression was occurring. As shown in Figure 6.3A, B, 8/10 C-terminal polyhistidine and 6/10 N-terminal tagged proteins were detected using Coomassie stained SDS-PAGE. The supernatant was subsequently tested against antigen by ELISA. Figure 6.4C reveals the N-polyHis proteins displayed lower binding to antigen than the C-polyHis proteins. There is a caveat here in that, this was performed with supernatant and from the SDS-PAGE gel (Figure 6.4A,B) it is observed that some constructs expressed less than others. Naturally, this would translate to lower binding on ELISA due to less protein. However, in proteins that were similarly expressed, for example C-terminal 2.4 and N-terminal 1.4, there was a significant difference in binding to MDA-LDL (Figure 6.4C). Lastly, it was observed that the N-polyHis proteins were not detectable by western blot (Figure 6.4D) while C-polyHis proteins were (Figure 6.4D). This led to the possibility that the N-polyHis proteins were binding to MDA-LDL but not detectable by the secondary antibody due to steric hindrance. However, it may also be that the N-polyHis tag interferes with the ABS itself, preventing binding and hence causing low to no signal. Lastly, the N-polyHis tag may be interfering with the folding and stability of the protein itself and thereby rendering it unable to bind antigen. If the N-polyHis tag was interfering with folding and stability this would also help to explain the rapid decline in viability and viable cell concentration of the N-polyHis tagged proteins in both HEK and Expi293 cells (Figure 6.2B, 6.3B). Nonetheless, the N-polyHis tagged proteins displayed lower binding (and expression) than C-polyHis tagged constructs and were not taken forward.

It is interesting that Goel et al. (2000) found the opposite where C-polyHIS scFv showed decreased binding compared to N-polyHis scFv. Thus, demonstrating the importance of determining which is better for every scFv as conclusions from one antibody are not necessarily transferable to another.

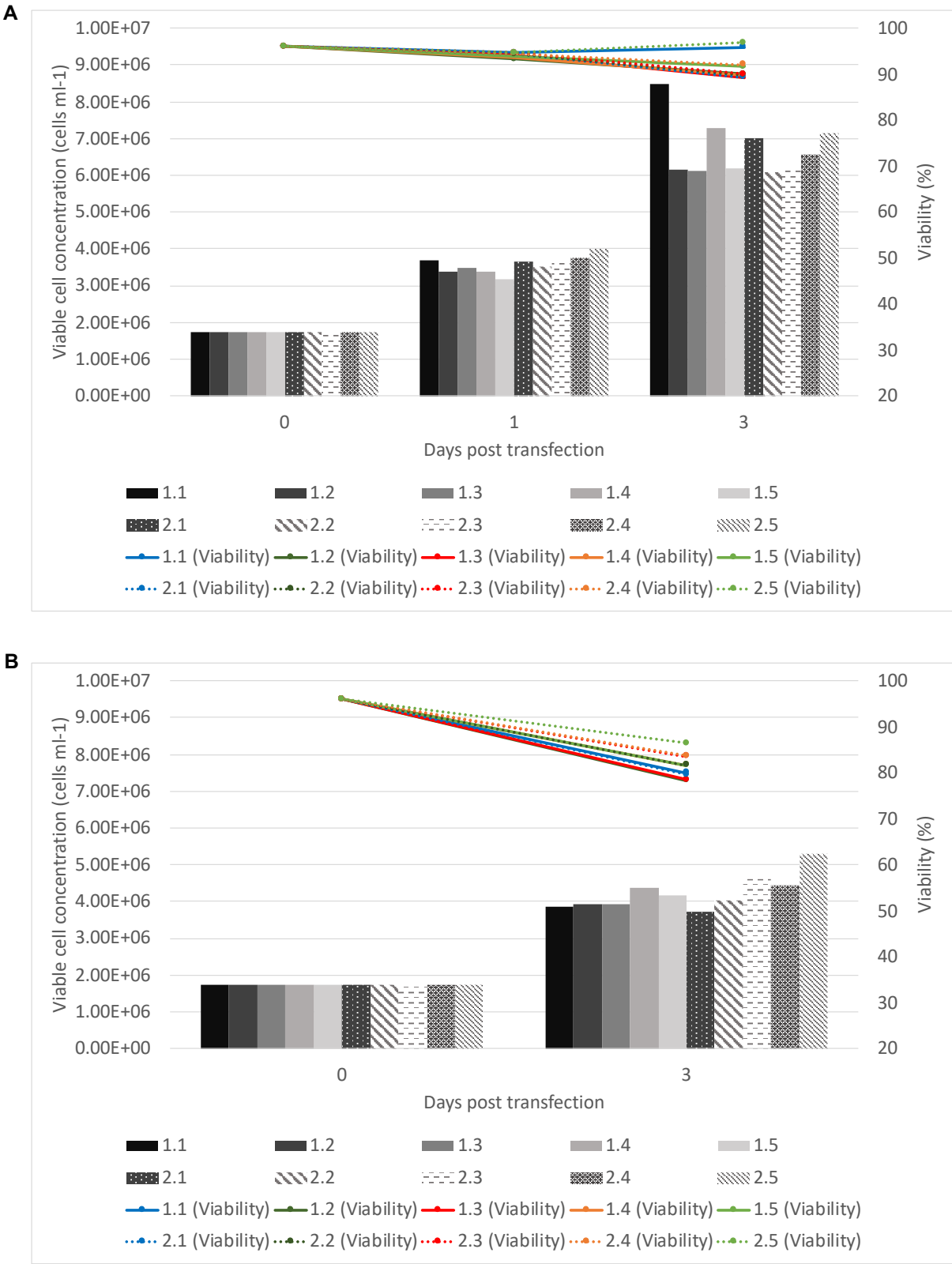


Figure 6.3: Expression of scFv variants in Expi293 cells. A) The viability (lines) and viable cell concentration (bars) of C-terminal polyhistidine tagged LO1 scFv constructs. Expression in HEK293 cells in 20 millilitres of Expi293 media. B) The viability (lines) and viable cell concentration (bars) of N-terminal polyhistidine tagged LO1 scFv constructs. Expression in HEK293 cells in 20 millilitres of Expi293 media.

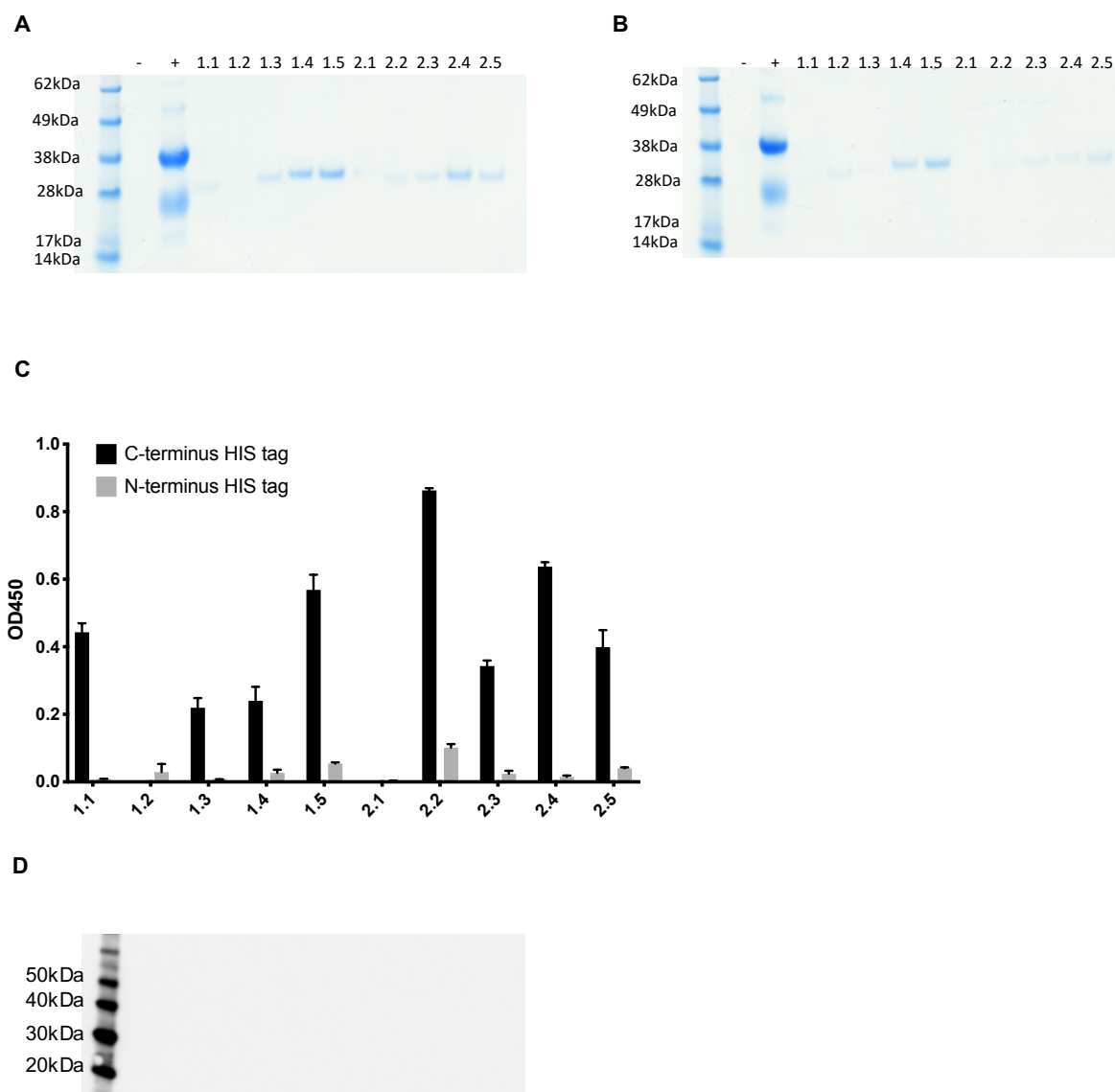


Figure 6.4: Polyhistidine tag on N or C terminus of LO1-scFv. A) Purified LO1-scFv - C terminal polyhistidine tagged. Negative control is cells transfected with no DNA. Positive control is cells transfected with a non-target scFv. B) Purified LO1-scFv – N terminus poly histidine tagged. Negative control is cells transfected with no DNA. Positive control is cells transfected with a non-target scFv. C) ELISA of purified C and N terminus polyhistidine tagged LO1-scFv constructs against $10 \mu\text{g ml}^{-1}$ of MDA-LDL. Values are mean $\pm$ SEM of technical replicates. D) Western blot of N-terminus polyhistidine tagged scFv using an anti-HIS secondary antibody.

6.2.3 Determining the orientation and linker length for lead LO1 scFv and diabody

It is well established that the orientation, VH-VL or VL-VH, is an important design aspect for the generation of scFv. Studies have been successful using both orientations, with the VH-VL

orientation more commonly employed (Yusakul et al. 2016, Liu et al. 2015, Bie et al. 2010). The linker length required may also change depending on the orientation as the distance between C-terminus of the VL and N-terminus of VH is larger than the distance between the C-terminus of VH and the N-terminus of VL (Huston et al. 1991, Arndt et al. 1998). Due to this, it has been suggested that VL-VH scFvs are more constrained than the VH-VL orientation when fused using the same linker and may form higher molecular weight oligomers more readily (Kortt et al. 1997, Dolezal et al. 2000). It is also reported that the orientation may impact the yields of the scFv with Tsumoto et al. (1994) and Hamilton et al. (2001) finding higher expression in the VL-VH orientation. On the other hand, Hamilton et al. (2001) found no change in binding and Tsumoto et al. (1994) found lower binding in this orientation.

The purified scFv proteins were investigated using western blot using an anti-His antibody. This showed two bands, with one at ~ 50 kda and the other at ~ 29 kDa (Figure 6.5A). The molecular weights indicate that the lower band at 29kda is likely to be monomeric scFv while the higher band is likely to be dimeric scFv. Upon reduction, the dimer was reduced indicating that the monomer was most likely covalently associating to form these dimers (Figure 6.5A,B). In this case, it is likely to be due to the engineered unpaired cysteine residue incorporated for fluorophore conjugation. Thus, it may not be a true diabody in having two ABS from the anti-parallel association of two scFv.

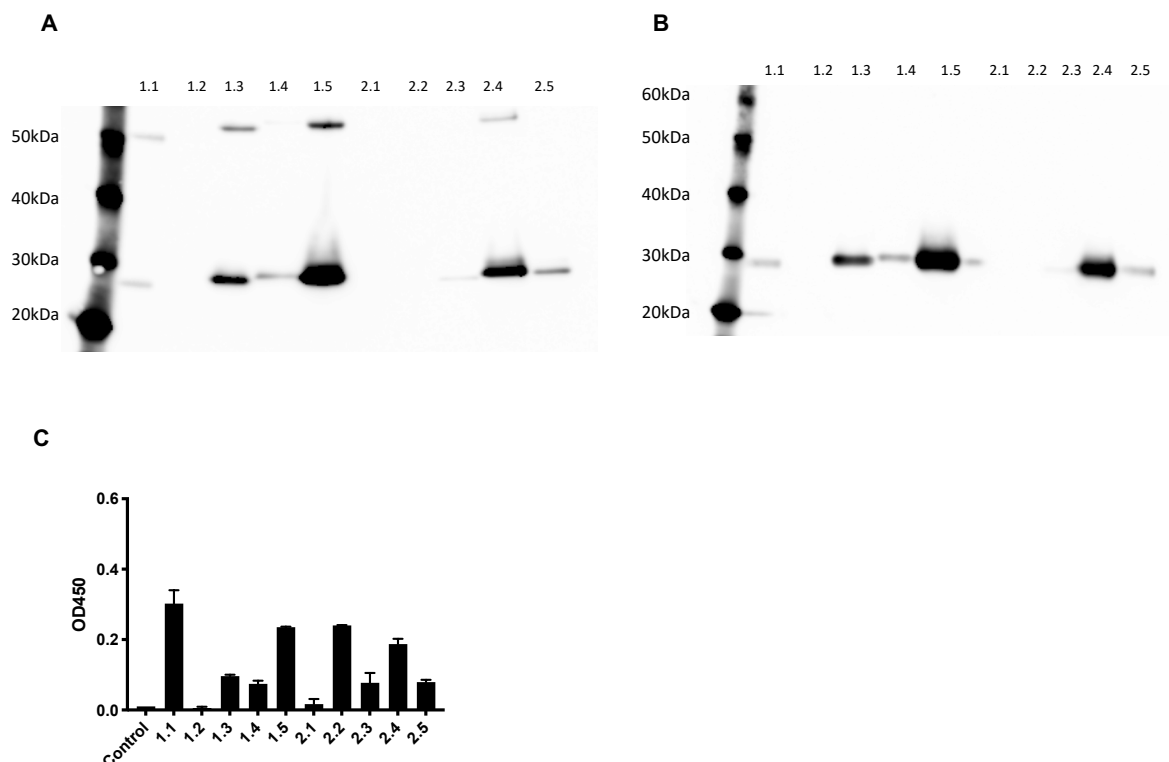


Figure 6.5: Expression of scFv variants in Expi293 cells. A) Western blot showing C-terminus polyhistidine tagged LO1-scFv proteins under non-reducing conditions. B) Western blot showing C-terminus polyhistidine tagged LO1-scFv proteins under reducing conditions. C) ELISA of $10\mu\text{g}$ purified C-terminus polyhistidine tagged LO1-scFv constructs against $10\mu\text{g ml}^{-1}$ of MDA-LDL. Control is a non-target His-tagged protein. Values are mean $\pm$ SEM of technical replicates.

Interestingly, for the VH-VL orientation 4/5 protein constructs were detected via western blot, while for the VL-VH orientation only 3/5 were detected (Figure 6.5A,B). Proteins with the $(\text{G}_4\text{S})_3$ linker (constructs 1.4 and 2.4) appeared to yield more protein in the VL-VH orientation. Additionally, the proteins with longer linkers appeared to express better regardless of orientation (1.4, 1.5, 2.4, 2.5). These constructs also appeared to be more highly expressed than proteins with shorter linker scFvs. The proteins with the shortest linkers $(\text{G}_4\text{S})_1$ and $(\text{G}_4\text{S})_2$ were expressed at low yield in the VL-VH orientation (2.1, 2.2) and expressed in the VH-VL orientation for only $(\text{G}_4\text{S})_1$ linker (1.1). Equal amounts of each protein were tested for binding against MDA-LDL using ELISA. This demonstrated that 1.1, 1.5 and 2.2 had the highest binding to antigen (Figure 6.5C). It was especially surprising that 2.2 bound well as it was not detectable by western blot indicating a low expression of this protein (Figure 6.5A,B). Non-reduced scFv demonstrated a $\sim 50:50$ split between monomer and dimer and thus its higher signal on ELISA may be due to

an avidity effect with the dimer (Figure 6.5C). This increase in signal was not entirely replicated with other constructs which displayed dimers such as 1.3 or 2.4. However, the proportion of dimer in these variants also appears to be lower which may account for this. Unfortunately, it was not possible to take all 10 constructs forward for further analysis. Therefore, protein constructs 1.1 and 1.5 were taken forward for further analysis due to their higher binding (1.1) and high expression (1.5). It was also hoped that these two would be used for making a diabody with 1.1 displaying $\sim 50\%$ dimer in non-reduced gel and having the shortest linker of 5 amino acids only.

6.2.4 Characterisation of lead scFv constructs

Purification

To further characterise the two lead scFv antibodies including understanding the impact of avidity on binding, the expression of the two lead scFvs was scaled up to gather enough protein. Upon purification of large scale (1L or 3L) expression, the His-NTA resin was stripped of its blue colour, indicating loss of the nickel from the resin, and the yield was significantly lower than expected. The Expi293 media used for expression of contains chelators which strip the nickel from traditionally used nickel columns. Cation exchange chromatography (CEX) was performed, making the scFvs positively charged acting as a first purification step and removing chelators from the buffer. Following CEX, immobilised metal affinity chromatography (IMAC) was performed using His-NTA resin and finally concentrated. This yielded low recoveries from the process (Figure 6.6A).

Finally, a different nickel resin, Ni-Sepharose Excel resin, was trialled. This yielded a definite strong band after purification (Figure 6.6B). This resin contains a chelating ligand for chelators in Expi293 media and related media which strip traditional nickel columns. Furthermore, the advantage of a one-step purification system limits losses which may explain the low yield recovery from CEX. It will later be shown that the overall yields of the scFv antibodies were very low,

and thus it is likely that a multi-step purification was leading to additional losses at each step, compounding the low expression resulting in low yield of purified protein.

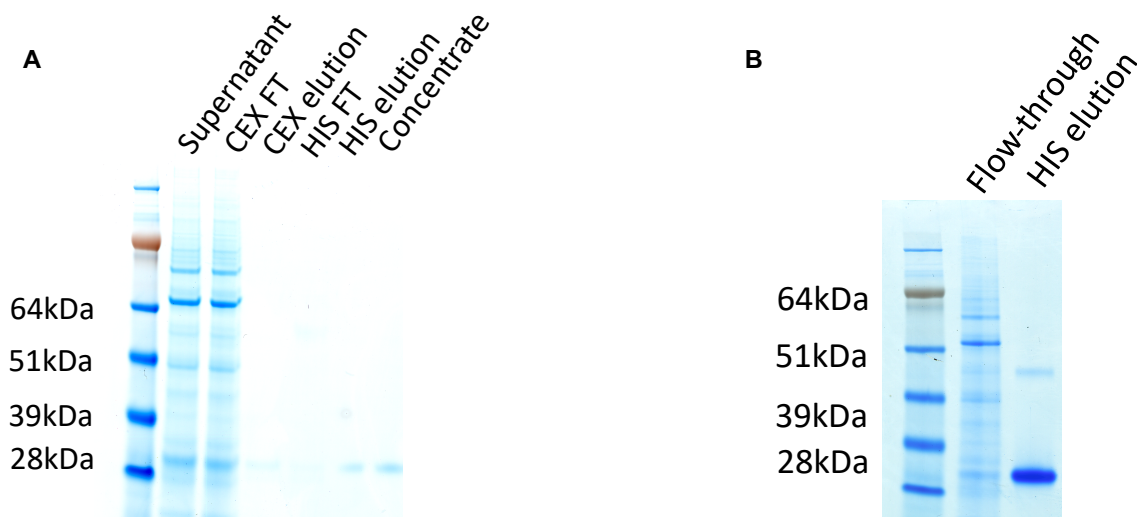


Figure 6.6: Developing a purification strategy for large scale scFv expression. A) Coomassie stained SDS-PAGE gel of variant 1.5 following cation exchange chromatography, purification using His-NTA resin and concentrating using Amicon centrifugal filter. B) Coomassie stained SDS-PAGE gel of variant 1.5 following purification using Ni-Sepharose Excel resin.

Separation of monomer from dimer

An aim of this thesis was to investigate the effect of avidity on antibody fragments of the same size, such as a Fab and diabody. It was planned for the diabody to be generated via the anti-parallel association of scFvs, thereby forming two binding sites. After expression of the scFv, dimer and monomer peaks of the scFv were separated using SEC (Figure 6.7A, B). On the principle of SEC, aggregates and higher molecular weight proteins such as the dimer would elute earlier than a monomer. Fractions from different peaks were collected and run on SDS-PAGE gel to determine the peaks. For both variants, multiple peaks were observed indicating the presence of aggregates and low molecular weight contaminants. The peaks corresponding to the monomer and dimer sizes contained both species within the collected fractions when run on Coomassie

SDS-PAGE gel (Figure 6.7C,D). It is hypothesised that the reason for both species in the peaks is insufficient resolution, where the peaks are not completely separated at the base. For both variants, a baseline was not reached between monomer and dimer peaks. Another possibility is a dynamic equilibrium between the two species. As the dimer is formed from non-covalent interactions, higher concentrations may induce dimer formation, while lower concentrations may help form scFv. Lee et al. (2002) described an anti-carcinoembryonic antigen scFv which dissociated and reassociated to form dimers in a concentration and time-dependent manner. Lastly, a diabody is generated due to steric reasons where an ABS and hydrophobic patches are only able to associate when two scFvs are in anti-parallel fashion. The VH-VL interface may be quite unstable resulting in fractions with both species. For the reasons listed above, diabodies produced in this manner are no longer preferred for *in vivo* work. Alternative methods of generating diabody include a linker between the two scFvs to maintain their fusion.

Despite trialling different protocols and columns, it was difficult to separate the two species. Additionally, the low yield of both scFv variants was requiring large culture volumes to gain minimum quantities of protein to use the SEC columns to get sufficient resolution. Due to large culture volumes, this required the supernatant to be concentrated by tangential flow filtration prior to SEC, adding additional steps that were prone to loss of protein. From a 1L expression, only 0.6mg of protein was recovered for variant 1.1 and 1.9mg for variant 1.5.

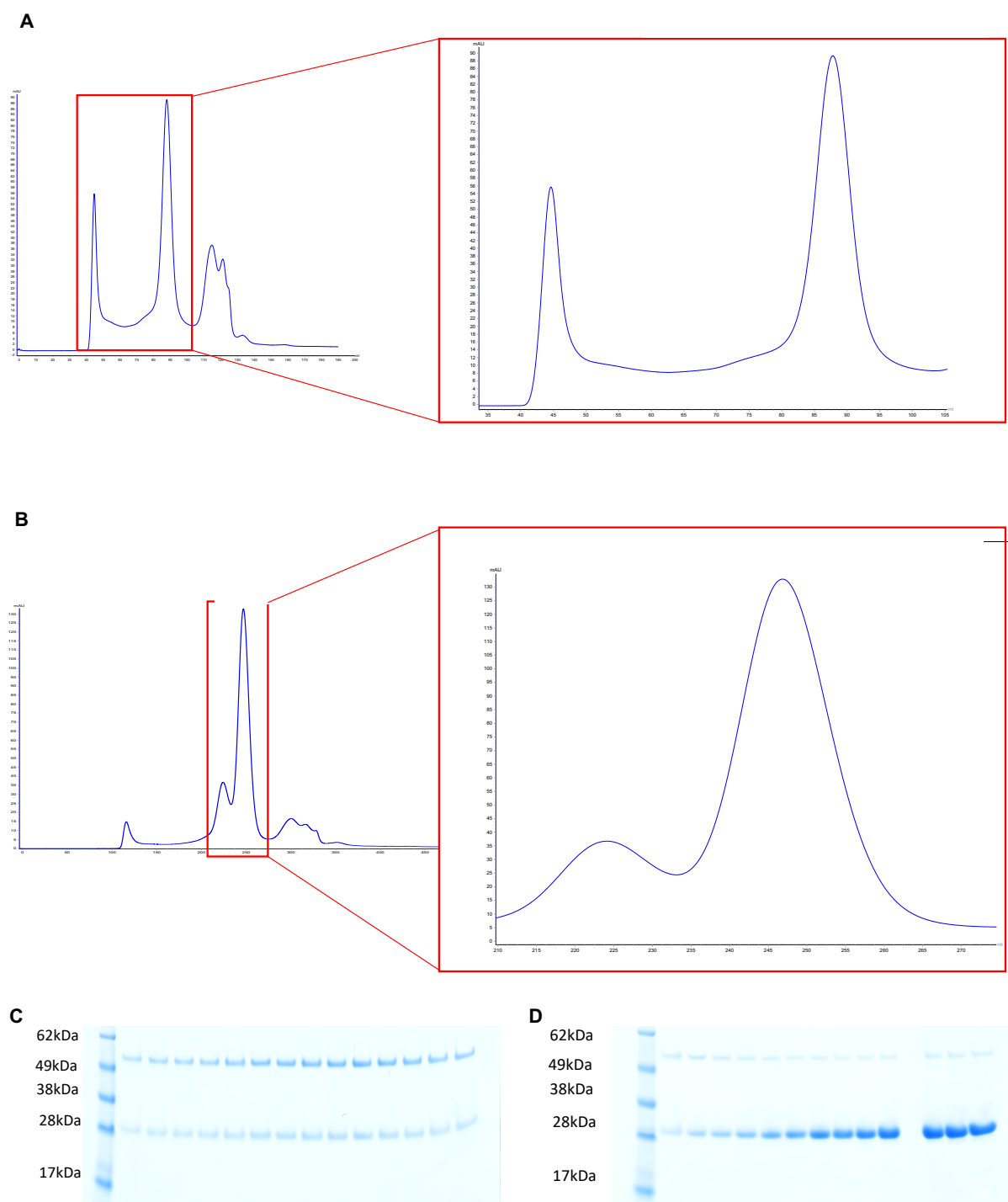


Figure 6.7: Dynamic equilibrium between monomer and dimer. A) Size exclusion chromatography of variant 1.1 B) Size exclusion chromatography of variant 1.5. C) Representative SDS-PAGE Coomassie stained gel of dimer peak from construct 1.5. D) Representative SDS-PAGE Coomassie stained gel of monomer peak from construct 1.5.

6.2.5 Generation of controls for lead constructs

From previous work, it was established that lysine residues in the LO1 sequence were important for binding to MDA-LDL (Khamis et al. 2016). It was also confirmed in Chapter 3 that the humanised Fab retained this property. When LO1-huFab was conjugated to NIR fluorophore using NHS chemistry, which reacts with lysine residues, it abrogated the binding to the antigen. This indicated that the lysine residues that are maintained in both the murine and humanised LO1 sequences are important for binding. 13 lysine residues fit this criterion, seven in the VH and six in the VL (Figure 4.5). The VH consists of two each in CDR2 and CDR3 and 3 in the frameworks. The VL consists of one each in CDR1 and CDR2 and 4 in the frameworks. It was hypothesised that the two lysine residues in CDR-H3 could be responsible for this loss of binding after fluorophore conjugation and therefore mutating them would provide good scFv controls. The two lysine residues in CDR-H3 were mutated to alanine (Figure 6.8A). Substitution with alanine residues eliminates side chain reactions without altering the main-chain conformation, and is the premise of alanine scanning which is utilised for epitope mapping. Two controls were generated, one for each variant. They were expressed in Expi293 and purified using Excel resin and demonstrated similar proportions of dimer and monomer to their relevant variant on SDS-PAGE (Figure 6.8B). These were subsequently tested on ELISA against MDA-LDL and showed significantly reduced binding to MDA-LDL (variant 1.1 vs 1.1-CDRH3-AA, $p < 0.0001$ and variant 1.5 vs 1.5-CDRH3-AA, $p = 0.0191$, Figure 6.8C). This confirms the importance of the two lysine residues in CDR-H3, indicating the binding to antigen can be almost completely abolished by the mutation of these two residues. This further confirms other studies about the importance of CDR-H3 for binding to antigen.

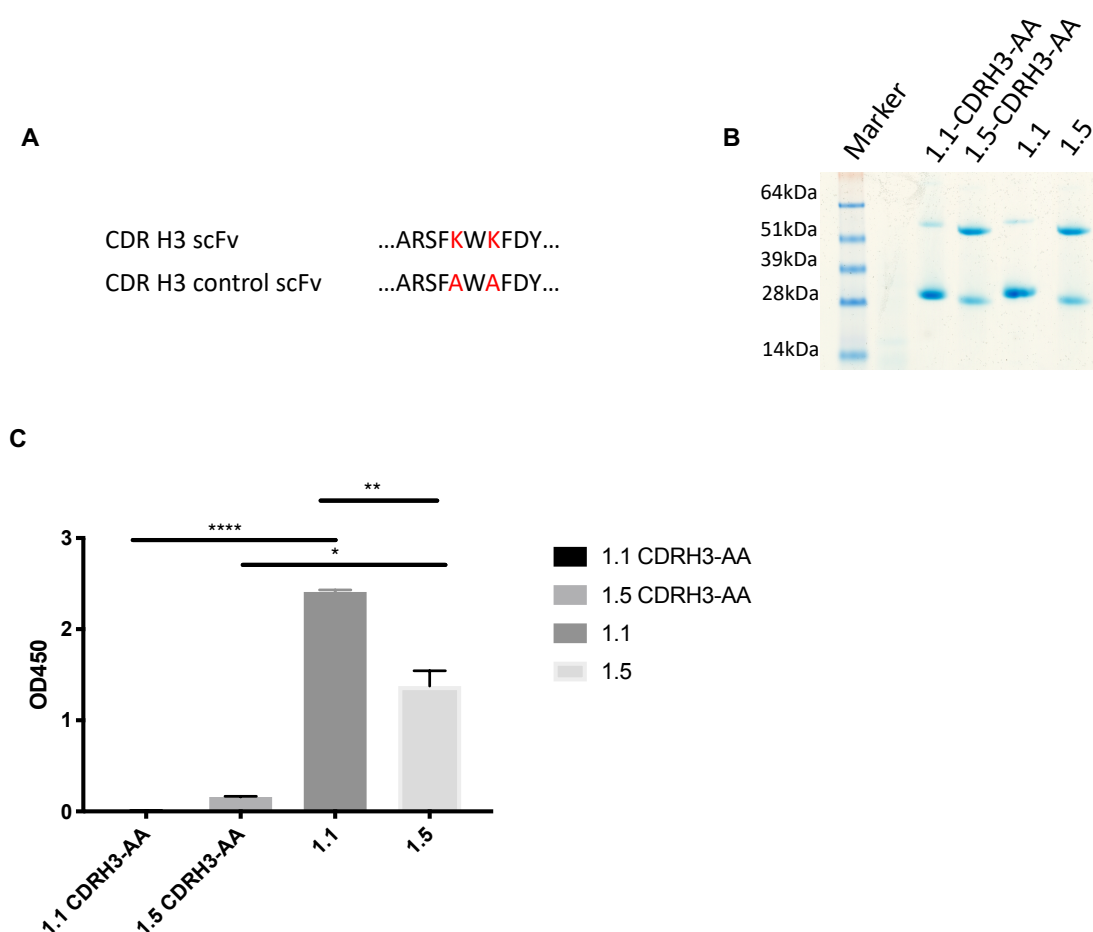


Figure 6.8: Generation of controls for lead constructs. A) Sequence alignment of CDR H3 region of murine scFv and control scFv where two lysine residues have been mutated to alanine. B) Coomassie stained SDS-PAGE gel of variants 1.1 and 1.5 and their respective controls, denoted by CDRH3AA. C) ELISA of lead scFv and controls against $10\mu\text{g ml}^{-1}$ of MDA-LDL. Error bars represent values $\pm$ SEM of technical replicates.

6.2.6 Immunohistochemistry of mouse aortic root

Similar to work in Chapter 3, immunohistochemistry was performed on serial sections of a LDLR^{-/-} mouse fed a HFD. The supplemented HFD accelerates atherosclerotic lesions within these mice and allows investigation into whether the scFv variants target MDA-LDL in a complex setting. Due to a limited amount of material, it was not possible to directly conjugate the scFv to NIR fluorophore. Rather, immunohistochemistry was performed using a secondary anti-HIS NIR fluorophore conjugated antibody. Interestingly, the variant 1.1 stained superficially on the lesion rather than within the necrotic core as previously experienced (Figure 6.9A,B). A few

serial sections away from these, it appears that the lesion had been damaged so this may be oxLDL that was released and coated the lumen of other sections (data not shown). This type of staining was not observed with its control, variant 1.1-CDRH3-AA, where no staining was visible (Figure 6.9E,F). Variant 1.5 shows staining within the core and in a similar location as variant 1.1 on the surface (Figure 6.9C,D). This type of staining was not observed with the control, where any signal also co-localised with autofluorescence (Figure 6.9G,H). For both variants 1.1 and 1.5, staining was present in areas which had reduced cellular content. This was similar to that seen before with both the parent antibody and the humanised Fab. Overall, on these sections, staining appeared to be lower than with the parent or humanised antibody. However, due to limited amounts of scFv it was not possible to check this on other serial sections and thus this interpretation would need to be validated on other sections and serial sections stained with parent and Fab to investigate if this were true.

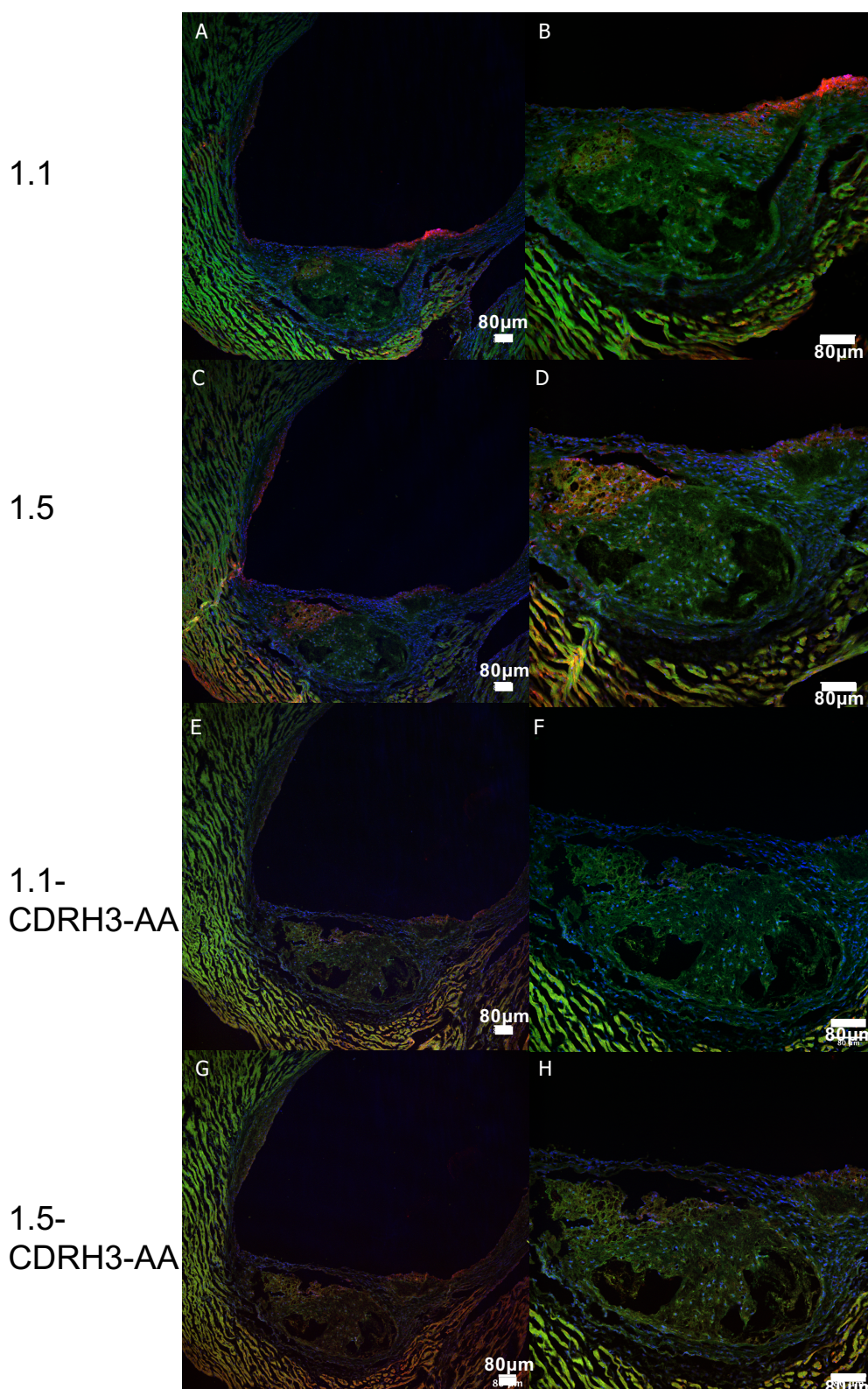


Figure 6.9: Immunohistochemistry of LO1 scFv. A), C), E), G) 10x confocal microscopy images of a LDLR^{-/-} fed high fat diet aortic root sections stained with variant 1.1, 1.5, 1.1-CDRH3-AA, and 1.15-CDRH3-AA. B), D), F), H), 20x confocal microscopy images of a LDLR^{-/-} fed high fat diet aortic root sections stained with variant 1.1, 1.5, 1.1-CDRH3-AA, and 1.15-CDRH3-AA. Red = scFV, green = autofluorescence, blue = 4'6-diamidino-2-phenylindole (DAPI).

6.2.7 Conclusion

The simple structure of scFv fragments allow for their easy manipulation. However, as experienced and described in this chapter, their practical use is more limited due to aggregation and low sample homogeneity. This chapter describes the generation and characterisation of LO1 scFv and diabody formats. It begins with the design and exploration of different linker lengths and compositions to generate well-expressed and functional scFvs. It was found that for the LO1 antibody sequence, a polyhistidine tag could only be implemented on the C-terminus as binding was not detected when attached to the N-terminus. Low expression and stability of the format resulted in difficulties during purification and gathering material for further *in vitro* and *in vivo* characterisation.

These difficulties are mediated by the low stability of the VH-VL interface. The interactions between the domains are generally weak and unlike in a Fab fragment where they are further stabilised by strong interactions in the CH1 and CL domains. There is a growing recognition and understanding that scFvs exist in an equilibrium between open and closed states. This dynamism causes the formation of dimers, trimer, and higher molecular weight aggregates (Arndt et al. 1998, Yamauchi et al. 2019). Open-state scFv, where the two domains do not form an ABS, leads to formation of oligomers. This in turn can increase avidity, but larger aggregates may result in precipitation. It was hoped that this oligomer formation would result in stable diabodies, however, as described in this chapter this was not possible. Several techniques have been described to overcome this difficulty with this small fragment, which largely revolve around increasing the interactions in the VH-VL interface. One of the methods involves the introduction of disulphide bonds into this interface (Röthlisberger et al. 2005). However, this is a long a tedious process as mutation sites must be worked out for each antibody. Additionally, structural data are very often required to effectively introduce these mutations. Another technique which has been described is the generation of cyclic scFvs, where, instead of increasing the VH-VL interface interaction, focus is put on preventing their dissociation by covalently connecting the C and N-terminuses (Yamauchi et al. 2019). By employing this method Yamauchi et al. (2019) found suppression of the open-closed dynamics without affecting binding affinity or stability.

Despite the difficulties associated with the scFv format, the first scFv, Brolucizumab, has recently been approved for clinical use (Sharma et al. 2020, Kaplon et al. 2020). Prior to this scFvs had only received clinical approval in other formats. One of which is the bispecific T-cell engager (BiTE) format, where two scFv fragments are fused using a linker. Blinatumomab is an FDA-approved bispecific T-cell engager for treating B-cell precursor lymphoblastic leukaemia. Each scFv binds a different antigen making it bispecific. A similar approach would be recommended for the generation of a LO1 diabody, with two LO1 VH and VL on one vector with a linker in between. Similarly to the approach described in this chapter, the linker in between the two scFv is kept short with Blinatumomab consisting of one repetition of a $(G_4S)_n$ linker. The linker maintains the association of these two scFvs while allowing free rotation of the two arms to allow for interactions with antigens. In humans, Blinatumomab has shown a half-life of 1.25 ± 0.63 hours which would be ideal for imaging (Portell et al. 2013).

In this chapter, expression was described in mammalian HEK cells. This approach was explored due to the reports that *E.coli* expressed scFvs may demonstrate improper folding (Vendel et al. 2012), avoid *in vitro* refolding processes and have endotoxin contamination. In addition, expression in HEK cells allows secretion of the antibody fragments into the culture supernatant. Outsourced production by a contract research organisation using the same protocol also yielded similar results, indicating HEK expression is not ideal for these scFv. These data indicate that for a murine LO1 scFv, an alternative host system may result in greater yields. However, it may also have resulted in misfolded protein as experienced by Vendel et al. (2012), where, when comparing scFv in CHO cells and *E. coli* it was discovered that while bacterial expressed scFv bound to the antigen, it was misfolded and had non-specific binding. Yusakul et al. (2016) also demonstrated that *E.coli* expressed scFv resulted in non-specific binding, which was not found when expressed in insect cells. Due to their aglycosylated nature, scFvs and diabodies can also be generated in *P. pastoris*, *E. coli* or insect cells. *P. pastoris* has an advantage of also being an endotoxin-free host system and as eukaryotic organism is capable of eukaryotic protein processing, folding and PTMs (Montoliu-Gaya et al. 2017). These alternative host systems may generate acceptable yields for in-depth characterisation. Another consideration

for the low yield may be that this unstable format is further perturbed by the presence of the unpaired cysteine for fluorophore conjugation. It is well accepted that an unpaired cysteine can reduce yield and may even impact antigen binding (Schmiedl et al. 2000, Banks et al. 2008).

Chapter 7

Concluding Remarks and Future work

7.1 Summary of results

This thesis has investigated the humanisation and reformatting of an anti-oxidised LDL antibody for the purposes of translating a scientific endeavour into a clinical one. The antibody humanisation and reformatting was designed in a rational manner to examine the effect of different residues and linkers to understand the impact they played on the both the antigen binding and the biophysical properties of the antibody formats themselves.

This thesis began with a literature review of the current understanding of antibody structure and the methods to generate humanised or fully human antibodies. It introduced modularity of antibodies and antibody formats relevant to the aim of this thesis. This was followed by a review of the pathology of atherosclerosis, its impact globally, and the importance of oxidised LDL in general, and MDA-LDL in particular, in its pathobiology. Subsequently, the literature review introduced molecular imaging as a method to tie these fields together. It described the various techniques available for targeted imaging and put forward an argument for NIR technology using an antibody against MDA-LDL. To conclude this chapter, the aims of the project were presented.

This thesis has demonstrated a method of humanising LO1 while simultaneously de-risking the molecule through the implementation of *in silico* sequence and structural analysis. On the whole, this found the humanised LO1 Fab' fragment to be a relatively safe molecule with no deamidation sites, aspartate isomerisation sites or regular cleavage sites. While this identified potential methionine and tryptophan oxidation sites, these were not explored experimentally. This *in silico* analysis highlighted residues that may increase immunogenic potential by introducing MHC or T-cell epitopes. This found that the backmutation of only three residues, Val67Ala, Ile69Leu, Tyr94Phe may increase the immunogenic potential. The humanised variants with the least immunogenic potential were explored further and their expression optimised to yield the greatest amount of full Fab' fragment. The lead Fab' fragment was characterised and found to retain binding to MDA-LDL and not plastic or native LDL. It was also found to retain binding at the same site as LO1 as a competitive ELISA demonstrated reduced binding with increasing concentrations of LO1. Excitingly, both parent LO1 and humanised Fab' demonstrated similar ability to capture MDA-LDL when coated at equal molar binding sites. This suggests the Fab' may have a similar affinity to the parent LO1, albeit with lower avidity. Following this preliminary *in vitro* investigation, *ex vivo* and *in vivo* characterisation was performed in diseased and wild-type mice demonstrating the ability of the humanised Fab' to distinguish arterial disease states. This study has revealed increased levels of NIR signal in the liver and kidneys. The liver signal may originate from homeostatic clearance or from tissue-specific targeting within the liver. Further studies with macrophage-specific markers may aid in distinguishing one from another. This study also raised the possibility of investigating other diseases such as NASH.

Hybrid modality imaging was employed through the combination of FMT with CT providing the benefit of improved quantification for reconstructions due to the anatomical coregistration. Currently, the use of NIRF imaging by the method described in this thesis is limited by the lack of clinically approved fluorophores for human use. Indocyanine green and is the only fluorophore approved by the FDA. While not having its own FDA approval, LiCor IRdye 800 CW has been conjugated to other approved therapies such as panitumumab and cetuximab and utilised in Phase 1 trials (Gao et al. 2018). As the cancer fields are overwhelmingly demonstrating an

added benefit for fluorescence guided surgery when resecting tumours, it is likely that in the foreseeable future, the same type of molecular investigation will arise for atherosclerosis and other diseases. A feasibility study was conducted using IRDye 800, but, unfortunately, available equipment to was unable to properly quantify the conjugation ratio of dye to antibody and thus would not allow calibration of the FMT to allow quantification of the amounts of localised antibody.

As far as we are aware this is only the second study to use IntegriSense as an imaging agent in studying preclinical atherosclerosis models. It has predominantly been used in cancers studies, due to ability to target vessels undergoing neovascularisation, a hallmark feature of cancer. Similarly, atherosclerotic plaques undergo neovascularisation in a homeostatic mechanism that may lead to disease progression and deleterious events such as intraplaque haemorrhages. While FMT and the NIRF field is currently limited by the availability of characterised probes, the use and characterisation of two well-studied probes in this study lends strength to the argument that LO1-huFab recognises atherosclerotic animals prior to IntegriSense and MMPSense. It also demonstrates the ability of molecular imaging to visualise MDA-LDL and may help bridge the gap of identifying vulnerable and stable plaques. Additionally, as far as we aware this is the first humanised antibody fragment utilised for the purposes of atherosclerosis imaging.

Interest in nanoparticle-based imaging of atherosclerosis has been growing. Even more so is the future potential of incorporating drug-eluting nanoparticles to targeting moieties such as the humanised Fab for specific delivery of drugs to plaques. A recent study by Hossaini Nasr et al. (2020), demonstrated the use of a hyaluronan-atorvastatin conjugate to reduce inflammation within a mouse model. Duivenvoorden et al. (2014) demonstrated the feasibility of using a statin-HDL nanoparticle to reduce macrophage content within plaques. Antibody-nanoparticle conjugates are likely to contribute to a sizeable portion of the theranostic field as they provide specificity, affinity and serum stability.

7.2 Limitations and suggestions for future studies

Challenges remain in the translation of animal studies to humans. Firstly, ApoE^{-/-} and LDLR^{-/-}, the most widely used atherosclerosis models, do not develop complex rupture-prone plaques similar to those in humans. Thus, it would be important to test the humanised Fab' in animal models which present with features characteristic of a clinical vulnerable plaque. For example, female ApoE^{-/-} mice with the Fbn1 gene mutation experience plaque rupture, intraplaque neovascularisation and haemorrhages (Emini Veseli et al. 2017). Additionally it is well established that genetic diversity plays a role in human disease. As most models are derived from a single mouse strain, it is difficult to capture the genetic diversity observed in patients.

It would be important and interesting to do further colocalisation studies using macrophage markers and angiogenic markers. It would also be important to test the Fab against other antigens, such as HDL, and extracellular matrix proteins to ensure its specificity to MDA-LDL.

Future studies on a murine LO1 scFv or diabody should evaluate alternative expression systems such as *E.coli* or *P. pastoris* as mentioned in Chapter 5. An investigation into the stability of the VH-VL interaction by adjusting the ionic strength, pH and the presence of antigen was planned, however was unable to be conducted due to the low amount of protein obtained. Furthermore, it is unlikely that an unlinked diabody would be suitable for *in vivo* studies and it would be ideal to add a linker and express two scFvs linked together.

Affinity measurement for a heterogenous target such as MDA-LDL has been difficult. However in order to proceed further down the translational pipeline, it is likely that this will need to be determined to ensure sufficient targeting ability. This may also result in the requirement of affinity maturation of the humanised antibody fragments as is often required after humanisation. This is commonly performed by PCR-directed site mutagenesis and screening for binders with higher affinity.

Both the scFv and humanised Fab have cysteine engineered for site-specific conjugation ensuring their structure is not disrupted. The current study demonstrated targeted fluorophore

conjugation using maleimide. Unfortunately, studies have demonstrated that maleimide linkers are susceptible to hydrolysis and may undergo thioether exchange with albumin, and therefore are not stable in human serum (Shen et al. 2012). This may lead to premature cleavage of the fluorophore or nanoparticle leading to off-target imaging or drug delivery. To overcome this, they must be hydrolysed post-conjugation, which may lead to reduction in yield of conjugated antibody, exposure to high pH conditions and long reaction times. Newer conjugation techniques include sulfone labelling chemistry, dibromomaleimide conjugation and click chemistry (Patterson et al. 2014, Wall et al. 2020, Oliveira et al. 2017). Sulfone labelling maintains chemoselectivity with both cysteine and selenocysteine, ensuring specific covalent conjugation and better stability than maleimide conjugation (Patterson et al. 2014).

Molecular diagnostic imaging techniques are increasingly being trialled for the implementation of personalised medicine. Recent trials in cancer with antibody-based molecular imaging have demonstrated the potential to aid diagnosis and improve outcomes. Bringing LO1-huFab based molecular imaging into to clinics will require the validation of low immunogenicity of the antibody upon repeated imaging using cell-based assays and relevant animal models. Validation of the modality in models with vulnerable plaques will be essential to gaining regulatory approval for Phase 1 clinical studies. It will also be important to gain insights into improving risk prediction of vulnerable plaques. While a key usage of this technology will be the diagnosis of rupture-prone plaques, it will also play a role in high-risk individuals for longitudinal monitoring of other therapies.

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Appendix A

Design of Antibody Fragments

[illegible]

Figure A.1: scFv geneblocks for cloning

[illegible]

Figure A.2: scFv geneblocks for cloning continued

Geneblock	DNA sequence
pDOM40-LO1_H0VH-Fab	tgctgtgggtgccccggatccacggcCAGGTGCGAGCTGGTGCAGAGCGGAGCCGAGGTGAAAGCCCGCAGCAGCGTGAAGGTGAGCTGCAAGGCCTCCGGCGGACCTTCAGCAACTACCTGATCGAGTGGGTGAGGCAGGCTCCCGGACAGGGCCTGGAGTGGGCTGATCAACCCCGCAGCGCGGCACCAACTACAGAGAAAGTTCAAGGGAAGGTTCAACATCACGCCGACAAAGAGCACCAGCCTACATGGAACTCAGCAGCTGAGGAGCGAGGACACCCCGCTGTACTATTGCGCAGGAGCTTCAAGTGAAGTTTCGACTACTGGGGCCAGGGCACCCCGTACTGTGAGCAGCGtagcacaagggcc
pDOM40-LO1_H1VH-Fab	tgctgtgggtgccccggatccacggcCAGGTGCGAGCTGGTGCAGAGCGGAGCCGAAAGTGAAGGCGGAAAGCCCGGAGCGGTGAAGGTGAGCTGCAAGGCCTCCGGGTACGCCCTTCACCAACTACCTGATCGAGTGGGTGAGGCAGGCCCCCGCCAGGGCCTGGAGTGGATGGGGTGTATCAACCCCGCAGCGCGGCACCAACTACACGAGAAAGTTCAAGGCGAGGTGACCATCAAGCCGCAAGCAAGCACCAGCAGCCTACATGGAGCTGAGCAGCTGAGGAGCGAGGACACCCCGTGTATTACTGCGCAAGGAGCTTCAAGTGAAGTTTCGACTACTGGGGCCAGGGCACCCCGTGACCGTGTGAGCTCTgttagcacaagggcc
pDOM40-LO1_H2VH-Fab	tgctgtgggtgccccggatccacggcCAGGTGCGAGCTGGTGCAGAGCGGAGCCGAAAGTGAAGGCGGAAAGCCCGGAGCGGTGAAGGTGAGCTGCAAGGCCTCCGGGTACGCCCTTCACCAACTACCTGATCGAGTGGGTGAGGCAGGCCCCCGCCAGGGCCTTGAGTGGGCTGATCAACCCCGCAGCGCGGCACCAACTACAGAGAAAGTTCAAGGGCAGGGCCACCTGACAGCCGACAGAGCACCAGCCTACATGGAGCTGAGCAGCCTGAGGAGCGAGGACACCCCGTGTATTACTGCGCAAGGAGCTTCAAGTGAAGTTTCGACTACTGGGGCCAGGGCACCCCGTGACCGTGTGAGCTCTgttagcacaagggcc
pDOM40-LO1_H3VH-Fab	tgctgtgggtgccccggatccacggcCAGGTGCGAGCTGGTGCAGAGCGGAGCCGAAAGTGAAGGCGGAAAGCCCGGAGCGGTGAAGGTGAGCTGCAAGGCCTCCGGGTACGCCCTTCACCAACTACCTGATCGAGTGGGTGAGGCAGGCCCCCGCCAGGGCCTGGAGTGGATGGGGTGTATCAACCCCGCAGCGCGGCACCAACTACACGAGAAAGTTCAAGGCGAGGTGACCATCAAGCCGCAAGCAAGCACCAGCAGCCTACATGGAGCTGAGCAGCTGAGGAGCGAGGACACCCCGTGTATTCTGCGCAAGGAGCTTCAAGTGAAGTTTCGACTACTGGGGCCAGGGCACCCCGTGACCGTGTGAGCTCTgttagcacaagggcc
pDOM40-LO1_H4VH-Fab	tgctgtgggtgccccggatccacggcCAGGTGCGAGCTGGTGCAGAGCGGAGCCGAAAGTGAAGGCGGAAAGCCCGGAGCGGTGAAGGTGAGCTGCAAGGCCTCCGGGTACGCCCTTCACCAACTACCTGATCGAGTGGGTGAGGCAGGCCCCCGCCAGGGCCTTGAGTGGGCTGATCAACCCCGCAGCGCGGCACCAACTACAGAGAAAGTTCAAGGGCAGGGCCACCTGACAGCCGACAGAGCACCAGCCTACATGGAGCTGAGCAGCCTGAGGAGCGAGGACACCCCGTGTATTCTGCGCAAGGAGCTTCAAGTGAAGTTTCGACTACTGGGGCCAGGGCACCCCGTGACCGTGTGAGCTCTgttagcacaagggcc

Figure A.3: Fab heavy chain geneblocks for cloning

[illegible]

Figure A.4: Fab light chain geneblocks for cloning

Appendix B

Peptide mass fingerprinting of humanised Fab

CoverPage

PROLIMS REPORT:

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5/21/2019 9:11:58 AM

satty.k.borman@gsk.com

<http://prolims.gsk.com/Report.aspx?s=62680&adt=ge1%2c94643%2c94644%2c94645%2csam>

Page 1

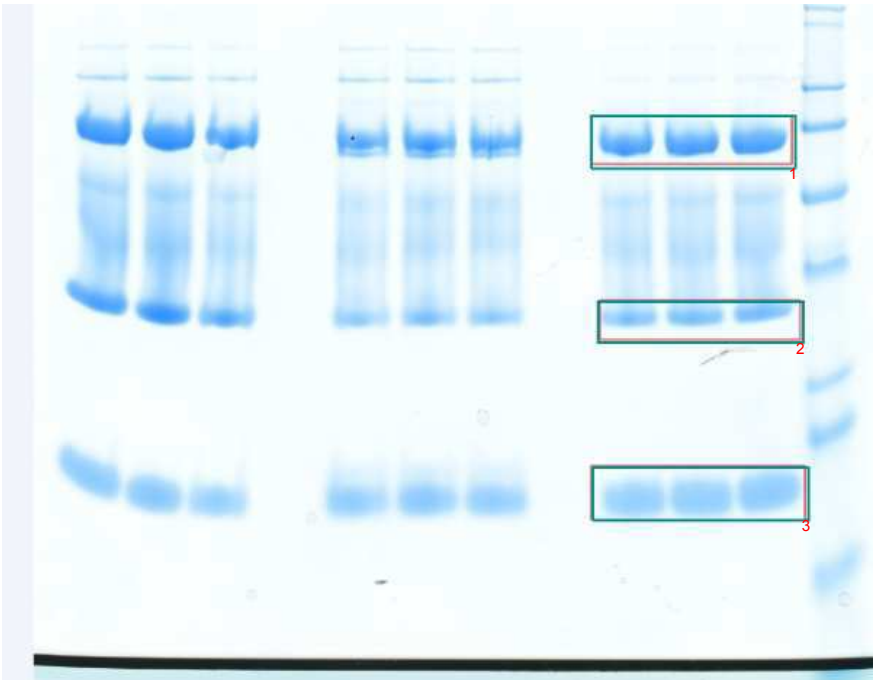


Analysis Report
Worldwide CSC and BR&AD

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LO1 Fab • ID # 62680

Submitted 17-May-2019 • Satty Borman
Project # 51659 • CD96 pathway antagonist mAb (i.v.) - cancer



Protein Identification ADV (Band 1 @ 525,104)

Completed 21-May-2019

Summary of Mascot Results / Analysis

Data File(s)	X:\Data\2019\Satty\Auto20May19\N72965-2-LO1\0_B1\1\1SRef\data\1\1r X:\Data\2019\Satty\Auto20May19\N72965-2-LO1\0_B2\1\1SRef\data\1\1r LTQ\Data\2019\Satty\20May19\N72965-2-LO1\200519_SP1T LTQ\Data\2019\Satty\20May19\N72965-2-LO1\200519_SP1C
Enzyme(s)	Trypsin, Chymotrypsin
Mascot DB(s)	GSK_PROLIMS
Mascot Searches	Mascot Search File - ../data/20190520/F3106338.dat Mascot Search File - ../data/20190521/F3106346.dat Mascot Search File - ../data/20190521/F3106348.dat

Acc. #	Protein ID	Pep. #	% Cov.	Score
13484	LO1-HUFAB_H1	n/a	71%	n/a
METDTLLLWV	LLLWVPGSTG	QVQLVQSGAE	VKKPGSSVKV	SKASGYAFT
NYLIEWVRQA	PGQGLEWMGV	INPGSGGTNY	NEKFKGRVTI	TADKSTSTAY
MELSSLRSED	TAVYYCARSF	KWKFDYWGQG	TTVTVSSAST	KGPSVFPLAP
SSKSTSGGTA	ALGCLVKDYF	PEPVTVSWNS	GALTSGVHTF	PAVLQSSGLY
SLSSVVTVP	SSLGTQTYIC	NVNHKPSNTK	VDKKVEPKSC	

Red = MS-MS Underline = PMF

Comments: Combined Sequence Coverage

13484	LO1-HUFAB_H1	30	51%	520
METDTLLLWV	LLLWVPGSTG	QVQLVQSGAE	VKKPGSSVKV	SKASGYAFT
NYLIEWVRQA	PGQGLEWMGV	INPGSGGTNY	NEKFKGRVTI	TADKSTSTAY
MELSSLRSED	TAVYYCARSF	KWKFDYWGQG	TTVTVSSAST	KGPSVFPLAP
SSKSTSGGTA	ALGCLVKDYF	PEPVTVSWNS	GALTSGVHTF	PAVLQSSGLY
SLSSVVTVP	SSLGTQTYIC	NVNHKPSNTK	VDKKVEPKSC	

Red = MS-MS Underline = PMF

Comments: Individual ions scores > 20 indicate identity or extensive homology (p<0.05).

Score	Peptide	Modifications	Obs. m/z	Delta	MS-MS?	PMF?
29	K.KPGSSVK.V		351.71	-0.0002	☒	☐
29	K.KPGSSVKVSCK.A		392.89	0.0005	☒	☐
(5)	K.KPGSSVKVSCK.A		392.89	0.0013	☒	☐
(16)	K.KPGSSVK.V		351.71	0.0007	☒	☐
(17)	K.KPGSSVK.V		351.71	0.0002	☒	☐
(23)	K.KPGSSVK.V		351.71	-0.0002	☒	☐
(23)	K.KPGSSVK.V		351.71	0.0003	☒	☐
71	K.ASGYAFTNYLIEWVR.Q		895.45	-0.0019	☒	☐
(61)	K.ASGYAFTNYLIEWVR.Q		895.45	0.0007	☒	☐
66	R.QAPGQGLEWMGVINPGSGGTNYNEK.F		1,302.61	-0.0045	☒	☐
16	K.GRVTITADK.S		480.78	-0.0006	☒	☐
(3)	K.GRVTITADK.S		480.78	-0.0014	☒	☐
23	R.VTITADK.S		374.22	0.0001	☒	☐
47	K.STSTAYMELSSLR.S	Oxidation (M)	731.35	-0.0002	☒	☐
72	R.SEDTAVYYCAR.S		667.79	-0.0002	☒	☐
12	R.SFKWK.F		348.20	0.0000	☒	☐
41	K.GPSVFPLAPSSK.S		593.83	-0.0017	☒	☐
(9)	K.GPSVFPLAPSSK.S		593.83	-0.0012	☒	☐
(30)	K.GPSVFPLAPSSK.S		593.83	-0.0019	☒	☐
66	K.STSGGTAALGCLVK.D		661.34	-0.0039	☒	☐
22	K.VDKKVEPK.S		314.86	0.0003	☒	☐
(5)	K.VDKKVEPK.S		471.78	-0.0003	☒	☐
(9)	K.VDKKVEPK.S		471.78	-0.0002	☒	☐
(11)	K.VDKKVEPK.S		471.78	-0.0003	☒	☐
(14)	K.VDKKVEPK.S		314.86	0.0008	☒	☐
(16)	K.VDKKVEPK.S		314.86	0.0000	☒	☐
(21)	K.VDKKVEPK.S		314.86	-0.0003	☒	☐

26	K.KVEPK.S		300.69	0.0002	☒	☐
(19)	K.KVEPK.S		300.69	0.0000	☒	☐
(22)	K.KVEPK.S		300.69	-0.0003	☒	☐

☐	13484	LO1-HUFAB_H1	29	60%	1029
☐	METDTLLLVW NYLIEWVRQA MELSSLRSED SSKSTSGGTA SLSSSVTVPS	LLLWVPGSTG PGQGLEWMGV TAVYYCARSF ALGCLVKDYF SSLGTQTYIC	QVQLVQSGAE INPGSGGTNY KWKFDYWGGG PEPVTVSWNS NVNHKPSNTK	VKKPGSSSVKV NEKFKGRVTI TTVTVSSAST GALTSGVHTF VDKKVEPKSC	SCKASGYAFT TADKSTSTAY KGPSVFPLAP PAVLQSSGLY

Red = MS-MS Underline = PMF

Comments: Individual ions scores > 20 indicate identity or extensive homology (p<0.05).

Score	Peptide	Modifications	Obs. m/z	Delta	MS-MS?	PMF?
42	L.VQSGAEVKKPGSSSVKVSCKASGY.A		785.08	0.0000	☒	☐
84	W.VRQAPGQGLEWMGVINPGSGGTNY.N		1,244.61	-0.0009	☒	☐
45	W.VRQAPGQGLE		463.26	-0.0016	☒	☐
(60)	W.VRQAPGQGLEWMGVINPGSGGTNY.N	Oxidation (M)	1,252.60	0.0018	☒	☐
65	L.EWMGVINPGSGGTNYNEKF.K		1,050.48	0.0016	☒	☐
(64)	L.EWMGVINPGSGGTNYNEKF.K	Oxidation (M)	1,058.48	0.0013	☒	☐
93	W.MGVINPGSGGTNYNEKF.K	Oxidation (M)	900.92	0.0006	☒	☐
56	W.MGVINPGSGGTNY.N		633.79	-0.0008	☒	☐
(26)	W.MGVINPGSGGTNYNEKF.K		892.92	0.0052	☒	☐
(64)	W.MGVINPGSGGTNYNEKF.K	Oxidation (M)	900.92	0.0019	☒	☐
(87)	W.MGVINPGSGGTNYNEKF.K		892.92	-0.0017	☒	☐
53	F.KGRVTITADKSTSTAY.M		849.95	0.0001	☒	☐
56	Y.MELSSLRSED TAVY.Y		800.88	-0.0027	☒	☐
(50)	Y.MELSSLRSED TAVY.Y	Oxidation (M)	808.88	-0.0006	☒	☐
53	L.SSLRSED TAVYY.C		695.83	-0.0015	☒	☐
31	L.SSLRSED TAVY.Y		614.30	-0.0024	☒	☐
32	Y.YCARSF.K		402.18	-0.0009	☒	☐
24	Y.CARSF.K		320.65	-0.0003	☒	☐
21	W.KFDYW.G		379.68	0.0004	☒	☐
64	L.APSSKSTSGGTAAL.G		617.82	-0.0021	☒	☐
67	L.GCLVKDYFPEPVTWSW.N		948.96	-0.0016	☒	☐
(39)	L.GCLVKDYFPEPVTWSW.N		948.96	0.0010	☒	☐
51	L.VKDYFPEPVTWSW.N		783.90	-0.0004	☒	☐
51	W.NSGALTSGVHTFPAVL.Q		785.92	0.0010	☒	☐
(22)	W.NSGALTSGVHTFPAVL.Q		785.92	0.0011	☒	☐
(29)	W.NSGALTSGVHTFPAVL.Q		785.91	-0.0002	☒	☐
(39)	W.NSGALTSGVHTFPAVL.Q		785.91	0.0002	☒	☐
89	L.TSGVHTFPAVLQSSGLY.S		882.45	-0.0006	☒	☐
52	L.TSGVHTFPAVL.Q		564.81	-0.0014	☒	☐

☐ 13485	LO1-HUFAB_L0	n/a	81%	n/a
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DIQMTQSPSSLSASVGDRVTITCKASENVGT YVSWYQQKPGKAPKLLIYG
ATKRYIGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCGQSYTYPYTFGG
GKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNFFYPREAKVQWKV
DNALQSGNSQESVTEQDSKDESTYLSSTLTLSKADYEKHKVYACEVTHQG
LSSPVTKSFNRGE

Red = MS-MSUnderline = PMF

Comments: Combined Sequence Coverage

13485LO1-HUFAB_L01276%51

DIQMTQSPSSLSASVGDRVTITCKASENVGT YVSWYQQKPGKAPKLLIYG
ATKRYIGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCGQSYTYPYTFGG
GKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNFFYPREAKVQWKV
DNALQSGNSQESVTEQDSKDESTYLSSTLTLSKADYEKHKVYACEVTHQG
LSSPVTKSFNRGE

Red = MS-MSUnderline = PMF

Comments: Individual ions scores > 27 indicate identity or extensive homology (p<0.05).

Score	Peptide	Modifications	Obs. m/z	Delta	MS-MS?	PMF?
0	-DIQMTQSPSSLSASVGDRVTITCK.A	Oxidation (M)	2,597.20	-0.0580	☐	☒
0	K.ASENVGT YVSWYQQKPGKAPK.L		2,338.15	-0.0364	☐	☒
0	K.LLIYGATKR.Y		1,034.64	0.0055	☐	☒
0	R.YIGVPSR.F		791.43	-0.0102	☐	☒
0	K.RTVAAPSVFIFPPSDEQLK.S		2,102.11	-0.0212	☐	☒
0	R.TVAAPSVFIFPPSDEQLK.S		1,946.01	-0.0178	☐	☒
51	K.SGTASVVCLLNFFYP.R		1,797.88	-0.0112	☒	☐
0	K.SGTASVVCLLNFFYP.R		2,126.04	-0.0269	☐	☒
0	K.VQWKVDNALQSGNSQESVTEQDSK.D		2,677.21	-0.0582	☐	☒
0	K.VDNALQSGNSQESVTEQDSKDESTYLSSTLTLSK.A		3,619.62	-0.0927	☐	☒
0	K.ADYKHKVYACEVTHQGLSSPVT.K		2,747.29	-0.0569	☐	☒
0	K.HKVYACEVTHQGLSSPVT.K		2,141.05	-0.0304	☐	☒

13485LO1-HUFAB_L03977%825

DIQMTQSPSSLSASVGDRVTITCKASENVGT YVSWYQQKPGKAPKLLIYG
ATKRYIGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCGQSYTYPYTFGG
GKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNFFYPREAKVQWKV
DNALQSGNSQESVTEQDSKDESTYLSSTLTLSKADYEKHKVYACEVTHQG
LSSPVTKSFNRGE

Red = MS-MSUnderline = PMF

Comments: Individual ions scores > 20 indicate identity or extensive homology (p<0.05).

Score	Peptide	Modifications	Obs. m/z	Delta	MS-MS?	PMF?
58	-DIQMTQSPSS.L		603.79	-0.0005	☒	☐
(44)	-DIQMTQSPSS.L	Oxidation (M)	611.78	-0.0011	☒	☐
41	L.SASVGDRVTITCKASENVGT.Y		739.03	-0.0008	☒	☐
(21)	L.SASVGDRVTITCKASENVGT.Y		739.03	-0.0001	☒	☐
(23)	L.SASVGDRVTITCKASENVGT.Y		739.03	0.0004	☒	☐
(25)	L.SASVGDRVTITCKASENVGT.Y		739.03	0.0006	☒	☐
(25)	L.SASVGDRVTITCKASENVGT.Y		739.03	0.0074	☒	☐
(27)	L.SASVGDRVTITCKASENVGT.Y		739.03	-0.0005	☒	☐
(30)	L.SASVGDRVTITCKASENVGT.Y		739.03	0.0013	☒	☐
(34)	L.SASVGDRVTITCKASENVGT.Y		739.03	-0.0007	☒	☐

(38)	L.SASVGDRVTITCKASENVGT.V	739.03	-0.0008	☒	☐
(40)	L.SASVGDRVTITCKASENVGT.V	739.03	-0.0016	☒	☐
(40)	L.SASVGDRVTITCKASENVGT.V	739.03	0.0048	☒	☐
28	W.YQKPGKAPKLL.I	685.91	-0.0003	☒	☐
31	Y.QQKPGKAPKLL.I	403.25	-0.0018	☒	☐
25	L.IYGATKRY.I	486.27	-0.0001	☒	☐
24	Y.GATKRY.I	348.20	-0.0004	☒	☐
30	Y.IGVPSRF.S	388.23	-0.0008	☒	☐
26	F.GGGTKVEIKRTVAAPSVF.I	909.02	0.0005	☒	☐
69	F.IFPPSDEQLKSGTASVVCLL.L	1,024.52	-0.0009	☒	☐
34	F.IFPPSDEQLKSGTASVVCLL.N	1,081.06	-0.0003	☒	☐
(22)	F.IFPPSDEQLKSGTASVVCLL.L	1,024.52	0.0008	☒	☐
(26)	F.IFPPSDEQLKSGTASVVCLL.N	1,081.06	-0.0005	☒	☐
(58)	F.IFPPSDEQLKSGTASVVCLL.L	1,024.52	0.0000	☒	☐
25	L.KSGTASVVCLL.L	511.27	-0.0016	☒	☐
21	L.KSGTASVVCLL.N	567.81	-0.0014	☒	☐
31	L.LNNFYPREAKVQW.K	832.93	-0.0003	☒	☐
(28)	L.LNNFYPREAKVQW.K	555.62	-0.0019	☒	☐
43	L.LNNFYPREAKVQW.K	776.39	0.0002	☒	☐
61	W.KVDNALQSGNSQESVTEQDSKDY.S	1,365.62	-0.0017	☒	☐
(54)	W.KVDNALQSGNSQESVTEQDSKDY.S	910.75	-0.0004	☒	☐
87	L.QSGNSQESVTEQDSKDY.S	1,045.44	0.0014	☒	☐
37	L.TLSKADYEKHKVY.A	791.42	0.0003	☒	☐
(26)	L.TLSKADYEKHKVY.A	527.95	-0.0026	☒	☐
24	L.SKADYEKHKVY.A	456.57	-0.0018	☒	☐
50	Y.ACEVTHQGLSPVTKSF.N	924.45	0.0003	☒	☐
46	Y.ACEVTHQGL.S	507.74	-0.0020	☒	☐
(42)	Y.ACEVTHQGLSPVTKSF.N	616.64	-0.0022	☒	☐
34	L.SSPVTKSF.N	426.73	-0.0016	☒	☐

☐ 13485 LO1-HUFAB_L0 79 76% **948**
☐ **DIQMTQSPSSLSASVGDRVTITCKASENVGTYSWYQQKPGKAPKLLIYG**
ATKRYIGVPSRFSGSGSGTDFTLTISLLQPEDFATYYCGQSYTYPTFGG
GTKVEIKRTVAAPSVFI FPPSDEQLKSGTASVVCLLNNFYPREAKVQWKV
DNALQSGNSQESVTEQDSKDYSLSLSTLTLSKADYEKHKVYACEVTHQG
LSSPVTKSFNRGE

Red = MS-MS Underline = PMF

Comments: Individual ions scores > 20 indicate identity or extensive homology (p<0.05).

Score	Peptide	Modifications	Obs. m/z	Delta	MS-MS?	PMF?
85	-.DIQMTQSPSSLSASVGDR.V	Oxidation (M)	947.94	-0.0001	☒	☐
19	R.VTITCK.A		361.20	0.0004	☒	☐
33	K.ASENVGTYSWYQQKPGKAPK.L		780.07	0.0021	☒	☐
20	K.ASENVGTYSWYQQKPGK.A		681.34	-0.0007	☒	☐

0	K.ASENVGTYSWYQQKPGKAPK.L		585.30	-0.0052	☒	☐
(1)	K.ASENVGTYSWYQQKPGKAPK.L		780.07	-0.0001	☒	☐
(2)	K.ASENVGTYSWYQQKPGKAPK.L		780.07	0.0039	☒	☐
(4)	K.ASENVGTYSWYQQKPGKAPK.L		585.30	-0.0001	☒	☐
(7)	K.ASENVGTYSWYQQKPGK.A		681.34	0.0013	☒	☐
(13)	K.ASENVGTYSWYQQKPGKAPK.L		780.07	-0.0007	☒	☐
(14)	K.ASENVGTYSWYQQKPGK.A		681.34	-0.0012	☒	☐
(21)	K.ASENVGTYSWYQQKPGKAPK.L		780.07	0.0019	☒	☐
51	K.LLIYGATKR.Y		517.82	-0.0005	☒	☐
36	K.LLIYGATK.R		439.77	-0.0008	☒	☐
(14)	K.LLIYGATKR.Y		345.55	-0.0005	☒	☐
(16)	K.LLIYGATKR.Y		345.55	0.0011	☒	☐
(40)	K.LLIYGATKR.Y		517.82	-0.0007	☒	☐
25	K.RYIGVPSR.F		474.27	-0.0008	☒	☐
34	R.YIGVPSR.F		396.22	-0.0006	☒	☐
23	K.VEIKR.T		322.71	0.0000	☒	☐
(4)	K.VEIKR.T		322.71	0.0003	☒	☐
(18)	K.VEIKR.T		322.71	0.0003	☒	☐
(20)	K.VEIKR.T		322.71	0.0003	☒	☐
(21)	K.VEIKR.T		322.71	0.0005	☒	☐
33	K.RTVAAPSVFIFPPSDEQLK.S		1,051.57	0.0006	☒	☐
68	R.TVAAPSVFIFPPSDEQLK.S		973.52	0.0002	☒	☐
51	R.TVAAPSVFIFPPSDEQLKSGTASVCLLNFFPR.E		1,242.31	-0.0001	☒	☐
(26)	R.TVAAPSVFIFPPSDEQLKSGTASVCLLNFFPR.E		1,242.31	0.0069	☒	☐
(33)	R.TVAAPSVFIFPPSDEQLKSGTASVCLLNFFPR.E		1,242.31	-0.0026	☒	☐
(35)	R.TVAAPSVFIFPPSDEQLKSGTASVCLLNFFPR.E		1,242.31	0.0021	☒	☐
(46)	R.TVAAPSVFIFPPSDEQLK.S		973.52	-0.0005	☒	☐
(46)	R.TVAAPSVFIFPPSDEQLKSGTASVCLLNFFPR.E		1,242.31	0.0043	☒	☐
(50)	R.TVAAPSVFIFPPSDEQLKSGTASVCLLNFFPR.E		1,242.31	0.0010	☒	☐
(51)	R.TVAAPSVFIFPPSDEQLKSGTASVCLLNFFPR.E		1,242.31	-0.0005	☒	☐
52	K.SGTASVCLLNFFPR.E		899.45	0.0003	☒	☐
49	K.VQWKVDNALQSGNSQESVTEQDSK.D		893.10	0.0150	☒	☐
29	K.VQWKVDNALQSGNSQESVTEQDSKDYSLSTLSK.A		1,387.67	-0.0055	☒	☐
(12)	K.VQWKVDNALQSGNSQESVTEQDSKDYSLSTLSK.A		1,387.67	-0.0099	☒	☐
(23)	K.VQWKVDNALQSGNSQESVTEQDSKDYSLSTLSK.A		1,387.67	-0.0062	☒	☐
97	K.VDNALQSGNSQESVTEQDSK.D		1,068.49	0.0003	☒	☐
61	K.VDNALQSGNSQESVTEQDSKDYSLSTLSK.A		1,207.25	0.0214	☒	☐
(45)	K.VDNALQSGNSQESVTEQDSKDYSLSTLSK.A		1,207.24	0.0023	☒	☐
(52)	K.VDNALQSGNSQESVTEQDSKDYSLSTLSK.A		1,207.24	0.0009	☒	☐
40	K.ADYEKHKVYACEVTHQGLSSPVTK.S		687.59	-0.0018	☒	☐

29	K.ADYEKHK.V		445.72	-0.0002	✗	☐
(6)	K.ADYEKHKVYACEVTHQGLSSPVT.K.S		916.45	0.0004	✗	☐
(10)	K.ADYEKHK.V		445.72	-0.0009	✗	☐
(11)	K.ADYEKHKVYACEVTHQGLSSPVT.K.S		687.59	0.0011	✗	☐
(17)	K.ADYEKHK.V		445.72	-0.0001	✗	☐
(17)	K.ADYEKHKVYACEVTHQGLSSPVT.K.S		687.59	0.0043	✗	☐
(18)	K.ADYEKHKVYACEVTHQGLSSPVT.K.S		687.59	-0.0016	✗	☐
(18)	K.ADYEKHKVYACEVTHQGLSSPVT.K.S		687.59	-0.0006	✗	☐
(19)	K.ADYEKHKVYACEVTHQGLSSPVT.K.S		687.59	0.0004	✗	☐
(22)	K.ADYEKHKVYACEVTHQGLSSPVT.K.S		687.59	0.0026	✗	☐
(24)	K.ADYEKHKVYACEVTHQGLSSPVT.K.S		687.59	0.0050	✗	☐
(32)	K.ADYEKHKVYACEVTHQGLSSPVT.K.S		687.59	-0.0023	✗	☐
(39)	K.ADYEKHKVYACEVTHQGLSSPVT.K.S		687.59	-0.0036	✗	☐
44	K.HKVYACEVTHQGLSSPVT.K.S		714.37	0.0011	✗	☐
(12)	K.HKVYACEVTHQGLSSPVT.K.S		714.37	-0.0002	✗	☐
(13)	K.HKVYACEVTHQGLSSPVT.K.S		536.02	-0.0045	✗	☐
(14)	K.HKVYACEVTHQGLSSPVT.K.S		714.36	-0.0019	✗	☐
(15)	K.HKVYACEVTHQGLSSPVT.K.S		536.03	-0.0013	✗	☐
(17)	K.HKVYACEVTHQGLSSPVT.K.S		714.37	0.0005	✗	☐
(25)	K.HKVYACEVTHQGLSSPVT.K.S		714.36	-0.0013	✗	☐
(27)	K.HKVYACEVTHQGLSSPVT.K.S		714.36	-0.0008	✗	☐
(32)	K.HKVYACEVTHQGLSSPVT.K.S		714.36	-0.0057	✗	☐
(32)	K.HKVYACEVTHQGLSSPVT.K.S		536.03	-0.0013	✗	☐
(33)	K.HKVYACEVTHQGLSSPVT.K.S		714.37	0.0091	✗	☐
(43)	K.HKVYACEVTHQGLSSPVT.K.S		714.37	0.0000	✗	☐
69	K.VYACEVTHQGLSSPVT.K.S		938.47	0.0007	✗	☐
(21)	K.VYACEVTHQGLSSPVT.K.S		938.47	-0.0016	✗	☐
(23)	K.VYACEVTHQGLSSPVT.K.S		625.98	-0.0032	✗	☐
(24)	K.VYACEVTHQGLSSPVT.K.S		938.47	0.0006	✗	☐
(36)	K.VYACEVTHQGLSSPVT.K.S		938.47	0.0022	✗	☐
(37)	K.VYACEVTHQGLSSPVT.K.S		625.98	-0.0014	✗	☐
(38)	K.VYACEVTHQGLSSPVT.K.S		625.98	0.0003	✗	☐
(45)	K.VYACEVTHQGLSSPVT.K.S		938.47	0.0003	✗	☐
(60)	K.VYACEVTHQGLSSPVT.K.S		625.98	-0.0004	✗	☐
(66)	K.VYACEVTHQGLSSPVT.K.S		938.47	0.0008	✗	☐

Analysis Details

Supplemental Report	
Analyst Notes	LO1-HUFAB_L0 and LO1-HUFAB_H1 were identified.
Analysts	Borman, Satty [satty.k.borman@gsk.com]
Site	Stevenage UK

Analysis Report

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Ultraflex Extreme (801613) - ABI - 4000 Qtrap
OrbiTrap (501482B) - Thermo - LTQ Orbitrap

Protein Identification ADV (Band 2 @ 531,241)

Completed 21-May-2019

Summary of Mascot Results / Analysis

Data File(s)	X:\Data\2019\Satty\Auto20May19\N72965-2-LO1\0_A3\1\1SRef\data\1\1r X:\Data\2019\Satty\Auto20May19\N72965-2-LO1\0_C3\1\1SRef\data\1\1r LTQ\Data\2019\Satty\20May19\N72965-2-LO1\200519_SP2T LTQ\Data\2019\Satty\20May19\N72965-2-LO1\200519_SP2C
Enzyme(s)	Trypsin, Chymotrypsin
Mascot DB(s)	GSK_PROLIMS
Mascot Searches	Mascot Search File - ../data/20190520/F3106341.dat Mascot Search File - ../data/20190520/F3106342.dat Mascot Search File - ../data/20190521/F3106350.dat Mascot Search File - ../data/20190521/F3106352.dat

Acc. #	Protein ID	Pep. #	% Cov.	Score
13484	LO1-HUFAB_H1	15	35%	499
METDTLLLVV	LLLVWPGSTG	QVQLVQSGAE	VKKPGSSSVKV	SCKASGYAFT
NYLIEWVRQA	PGQGLEWMGV	INPGSGGTNY	NEKFKGRVTI	TADKSTSTAY
MELSSLRSED	TAVYYCARSF	KWKFDYWGQG	TTVTVSSAST	KGPSVFPLAP
SSKSTSGGTA	ALGCLVKDYF	PEPVTVSWS	GALTSGVHTF	PAVLQSSGLY
SLSSVVTVP	SSLGTQTYIC	NVNHKPSNTK	VDKKVEPKSC	

Red = MS-MS Underline = PMF

Comments: Individual ions scores > 21 indicate identity or extensive homology (p<0.05).

Score	Peptide	Modifications	Obs. m/z	Delta	MS-MS?	PMF?
54	W.VRQAPQGLEW.M		620.82	-0.0031	☒	☐
24	L.EWMGVINPGSGGTNYNEKF.K	Oxidation (M)	1,058.48	0.0004	☒	☐
93	W.MGVINPGSGGTNYNEKF.K		892.92	-0.0001	☒	☐
28	W.MGVINPGSGGTNY.N		633.79	-0.0015	☒	☐
(78)	W.MGVINPGSGGTNYNEKF.K	Oxidation (M)	900.91	0.0002	☒	☐
58	Y.MELSSLRSEDNAVY.Y	Oxidation (M)	808.88	0.0004	☒	☐
(54)	Y.MELSSLRSEDNAVY.Y		800.88	0.0004	☒	☐
49	L.SSLRSEDNAVY.C		695.83	-0.0001	☒	☐
28	L.SSLRSEDNAVY.Y		614.30	-0.0006	☒	☐
24	Y.CARF.K		320.65	-0.0007	☒	☐
40	L.VKDYFPEPVTVSW.N		783.90	0.0001	☒	☐
57	W.NSGALTSVHTFPAVL.Q		785.92	0.0009	☒	☐
44	L.TSGVHTFPAVLQSSGLY.S		882.45	0.0029	☒	☐
(27)	L.TSGVHTFPAVLQSSGLY.S		882.45	0.0017	☒	☐
(43)	L.TSGVHTFPAVLQSSGLY.S		882.45	0.0017	☒	☐

13485	LO1-HUFAB_L0	n/a	85%	n/a
DIQMTQSPSS	LSASVGDRT	ITCKASENVG	IYVSWYQKQ	GKAPKLLIYG
ATKRYIGVPS	RFSGSGSGTD	FTLTISLQ	EDFATYYCGQ	SYTYPTFGG
GIKVEIKRTV	AAPSVEIFPP	SDEQLKSGTA	SVVCLLNFFY	PBEAKVQWKV
DNALQSGNSQ	ESVTEQDSKD	STYSLSSILT	LSKADYEKHK	VYACEVIHQG
LSSPVTKSFN	RGE			

Red = MS-MSUnderline = PMF

Comments: Combined Sequence Coverage

13485	LO1-HUFAB_L0	17	78%	179		
DIQMTQSPSSLSASVGDRVTITCKASENVGTYV ATKRYIGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQGSYTPYTFGG GKVEIKRIVAAPSVFIEPPSDEQLKSGTASVVCLLNNFYPREAKVQWKV DNALQSGNSQESVTEQDSKDSITYSLSSLTILSKADYEKHKVYACEVTHQG LSSPVTKSFNRGE Red = MS-MSUnderline = PMF Comments: Individual ions scores > 28 indicate identity or extensive homology (p<0.05).						
Score	Peptide	Modifications	Obs. m/z	Delta	MS-MS?	PMF?
0	.DIQMTQSPSSLSASVGDRVTITCKASENVGTY.V		3,402.60	-0.0142	☐	☒
0	.DIQMTQSPSSLSASVGDRVTITCKASENVGTY.V	Oxidation (M)	3,418.59	-0.0183	☐	☒
56	L.SASVGDRVTITCKASENVGTY.V		2,215.07	0.0024	☒	☐
0	L.SASVGDRVTITCKASENVGTYVSW.Y		2,587.25	0.0039	☐	☒
0	W.YQKPGKAPKLL.I		1,370.83	0.0112	☐	☒
0	Y.QQKPGKAPKLL.I		1,207.78	0.0279	☐	☒
0	L.IYGATKRY.I		971.53	0.0035	☐	☒
0	Y.IGVPSRF.S		775.43	-0.0127	☐	☒
0	F.GGGTKVEIKRTVAAPSVF.I		1,817.04	0.0160	☐	☒
0	F.IFPPSDEQLKSGTASVVCL.L		2,048.04	0.0026	☐	☒
29	L.NNFYPREAKVQW.K		1,551.79	0.0226	☒	☐
0	F.YPREAKVQW.K		1,176.63	0.0158	☐	☒
0	W.KVDNALQSGNSQESVTEQDSKDSITY.S		2,730.23	-0.0084	☐	☒
0	L.TLSKADY.E		797.41	0.0055	☐	☒
0	L.TLSKADYEKHKVY.A		1,581.85	0.0199	☐	☒
94	Y.ACEVTHQGLSSPVTKSF.N		1,847.91	0.0184	☒	☐
0	Y.ACEVTHQGL.S		1,014.50	0.0314	☐	☒

13485

LO1-HUFAB_L0

15

74%

219

DIQMTQSPSSLSASVGDRVTITCKASENVGTYV
ATKRYIGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQGSYTPYTFEGG
GKVEIKRIVAAPSVFIEPPSDEQLKSGTASVVCLLNNFYPREAKVQWKV
DNALQSGNSQESVTEQDSKDSITYSLSSLTILSKADYEKHKVYACEVTHQG
LSSPVIKSFNRGE

Red = MS-MSUnderline = PMF

Comments: Individual ions scores > 27 indicate identity or extensive homology (p<0.05).

Score	Peptide	Modifications	Obs. m/z	Delta	MS-MS?	PMF?
0	.DIQMTQSPSSLSASVGDR.V	Oxidation (M)	1,894.87	-0.0123	☐	☒
0	.DIQMTQSPSSLSASVGDRVTITCK.A		2,581.23	-0.0347	☐	☒
0	.DIQMTQSPSSLSASVGDRVTITCK.A	Oxidation (M)	2,597.21	-0.0406	☐	☒
0	K.ASENVGTYVSWYQKPGK.A		2,041.98	-0.0179	☐	☒
0	K.ASENVGTYVSWYQKPGKAPK.L		2,338.16	-0.0260	☐	☒
0	K.LLIYGATKR.Y		1,034.64	0.0004	☐	☒
0	R.YIGVPSR.F		791.42	-0.0256	☐	☒

0	K.RTVAAPSVFIFPPSDEQLK.S		2,102.11	-0.0179	☐	☒
51	K.SGTASVCLNNFYPR.E		1,797.88	-0.0110	☒	☐
0	K.VQWKVDNALQSGNSQESVTEQDSK.D		2,677.23	-0.0412	☐	☒
0	K.VQWKVDNALQSGNSQESVTEQDSKDYSLSTLTLSK.A		4,160.93	-0.0837	☐	☒
0	K.VDNALQSGNSQESVTEQDSKDYSLSTLTLSK.A		3,619.64	-0.0688	☐	☒
69	K.ADYEKHKVYACEVTHQGLSSPVT.K.S		2,747.31	-0.0370	☒	☐
0	K.HKVYACEVTHQGLSSPVT.K.S		2,141.06	-0.0224	☐	☒
99	K.VYACEVTHQGLSSPVT.K.S		1,875.91	-0.0142	☒	☐

13485	LO1-HUFAB_LO	20	61%	663
DIQMTQSPSSLSASVGDRVTITCKASENVGTYSWYQQKPKGKAPKLLIYG ATKRYIGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCGQSYTYPYTFGG GKVEIKRTVAAPSVFIFPPSDEQLKSGTASVCLNNFYPREAKVQWKV DNALQSGNSQESVTEQDSKDYSLSTLTLSKADYEKHKVYACEVTHQG LSSPVTKSFN RGE				

Red = MS-MS Underline = PMF

Comments: Individual ions scores > 18 indicate identity or extensive homology (p<0.05).

Score	Peptide	Modifications	Obs. m/z	Delta	MS-MS?	PMF?
120	..DIQMTQSPSSLSASVGDR.V		939.95	0.0003	☒	☐
(113)	..DIQMTQSPSSLSASVGDR.V	Oxidation (M)	947.95	0.0019	☒	☐
22	R.VTITCK.A		361.20	0.0001	☒	☐
30	K.ASENVGTYSWYQQKPGK.A		1,021.50	-0.0014	☒	☐
(21)	K.ASENVGTYSWYQQKPGK.A		681.34	-0.0003	☒	☐
45	K.LLIYGATK.R		439.77	-0.0008	☒	☐
34	K.LLIYGATK.R.Y		517.82	-0.0006	☒	☐
32	K.RYIGVPSR.F		474.27	-0.0001	☒	☐
29	R.YIGVPSR.F		396.22	-0.0002	☒	☐
27	K.VEIKR.T		322.71	0.0001	☒	☐
(22)	K.VEIKR.T		322.71	-0.0004	☒	☐
58	R.TVAAPSVFIFPPSDEQLK.S		973.52	0.0003	☒	☐
(44)	R.TVAAPSVFIFPPSDEQLK.S		973.52	0.0013	☒	☐
61	K.VQWKVDNALQSGNSQESVTEQDSK.D		893.10	0.0004	☒	☐
104	K.VDNALQSGNSQESVTEQDSK.D		1,068.49	-0.0024	☒	☐
(47)	K.VDNALQSGNSQESVTEQDSK.D		712.66	0.0005	☒	☐
22	K.ADYEKHK.V		445.72	-0.0002	☒	☐
(21)	K.ADYEKHK.V		445.72	0.0004	☒	☐
79	K.VYACEVTHQGLSSPVT.K.S		938.47	0.0007	☒	☐
(58)	K.VYACEVTHQGLSSPVT.K.S		625.98	-0.0019	☒	☐

13485	LO1-HUFAB_LO	40	73%	912
DIQMTQSPSSLSASVGDRVTITCKASENVGTYSWYQQKPKGKAPKLLIYG ATKRYIGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCGQSYTYPYTFGG GKVEIKRTVAAPSVFIFPPSDEQLKSGTASVCLNNFYPREAKVQWKV DNALQSGNSQESVTEQDSKDYSLSTLTLSKADYEKHKVYACEVTHQG LSSPVTKSFN RGE				

Red = MS-MS Underline = PMF

Comments: Individual ions scores > 21 indicate identity or extensive homology (p<0.05).

Score	Peptide	Modifications	Obs. m/z	Delta	MS-MS?	PMF?
84	--DIQMTQSPSSL.S		603.79	-0.0006	☒	☐
(53)	--DIQMTQSPSSL.S	Oxidation (M)	611.78	-0.0014	☒	☐
53	L.SASVGDRVTITCKASENVGTY.V		739.03	-0.0010	☒	☐
(27)	L.SASVGDRVTITCKASENVGTY.V		739.03	0.0035	☒	☐
(29)	L.SASVGDRVTITCKASENVGTY.V		739.03	-0.0005	☒	☐
(34)	L.SASVGDRVTITCKASENVGTY.V		739.03	0.0001	☒	☐
(40)	L.SASVGDRVTITCKASENVGTY.V		739.03	0.0026	☒	☐
(45)	L.SASVGDRVTITCKASENVGTY.V		739.03	-0.0018	☒	☐
(45)	L.SASVGDRVTITCKASENVGTY.V		739.03	0.0012	☒	☐
25	W.YQKPGKAPKLL.I		685.91	-0.0004	☒	☐
38	L.IYGATKRY.I		486.27	-0.0011	☒	☐
22	Y.GATKRY.I		348.20	0.0004	☒	☐
51	Y.IGVPSRFSGSGSGTDF.T		785.88	-0.0006	☒	☐
38	Y.IGVPSRF.S		388.23	-0.0015	☒	☐
72	F.IFPPSDEQLKSGTASVVCL.L		1,024.52	0.0010	☒	☐
42	F.IFPPSDEQLKSGTASVVCL.N		1,081.06	0.0002	☒	☐
(22)	F.IFPPSDEQLKSGTASVVCL.L		1,024.52	0.0012	☒	☐
(63)	F.IFPPSDEQLKSGTASVVCL.L		1,024.52	-0.0007	☒	☐
28	L.KSGTASVVCL.L		511.27	-0.0004	☒	☐
24	L.KSGTASVVCL.N		567.81	-0.0010	☒	☐
45	L.NNFYPREAKVQW.K		776.39	0.0013	☒	☐
68	W.KVDNALQSGNSQESVTEQDSKDY.S		910.75	0.0007	☒	☐
(35)	W.KVDNALQSGNSQESVTEQDSKDY.S		910.75	0.0011	☒	☐
(58)	W.KVDNALQSGNSQESVTEQDSKDY.S		1,365.62	-0.0007	☒	☐
77	L.QSGNSQESVTEQDSKDY.S		1,045.44	0.0004	☒	☐
42	L.TLSKADYEKHKVY.A		527.95	-0.0008	☒	☐
37	L.TLSKADY.E		399.21	-0.0009	☒	☐
(28)	L.TLSKADYEKHKVY.A		527.95	-0.0026	☒	☐
(28)	L.TLSKADYEKHKVY.A		527.95	-0.0002	☒	☐
(30)	L.TLSKADYEKHKVY.A		527.95	-0.0021	☒	☐
(31)	L.TLSKADYEKHKVY.A		791.42	0.0017	☒	☐
(33)	L.TLSKADYEKHKVY.A		527.95	-0.0013	☒	☐
(36)	L.TLSKADYEKHKVY.A		527.95	-0.0010	☒	☐
(36)	L.TLSKADYEKHKVY.A		791.42	0.0009	☒	☐
26	L.SKADYEKHKVY.A		456.57	-0.0017	☒	☐
(23)	L.SKADYEKHKVY.A		684.35	-0.0012	☒	☐
59	Y.ACEVTHQGLSSPVTKSF.N		924.45	-0.0014	☒	☐
38	Y.ACEVTHQGL.S		507.74	-0.0012	☒	☐
(42)			616.64	-0.0032		

	Y.ACEVTHQGLSSPVTKSF.N				☒	☐
43	L.SSPVTKSF.N		426.73	-0.0012	☒	☐

Analysis Details

Supplemental Report	
Analyst Notes	LO1-HUFAB_L0 and LO1-HUFAB_H1 identified. LO1-HUFAB_L0 was potentially the major component. The sequence coverage for LO1-HUFAB_H1 was lower than for band 1.
Analysts	Borman, Satty [satty.k.borman@gsk.com]
Site	Stevenage UK
Instruments	Ultraflex Extreme (801613) - ABI - 4000 Qtrap OrbiTrap (501482B) - Thermo - LTQ Orbitrap

Protein Identification ADV (Band 3 @ 531,371)

Completed 21-May-2019

Summary of Mascot Results / Analysis

Data File(s)	X:\Data\2019\Satty\Auto20May19\N72965-2-LO1\0_B4\1\1SRef\data\1\1r X:\Data\2019\Satty\Auto20May19\N72965-2-LO1\0_B5\1\1SRef\data\1\1r LTQ\Data\2019\Satty\20May19\N72965-2-LO1\200519_SP3T LTQ\Data\2019\Satty\20May19\N72965-2-LO1\200519_SP3C
Enzyme(s)	Trypsin, Chymotrypsin
Mascot DB(s)	GSK_PROLIMS
Mascot Searches	Mascot Search File - ../data/20190520/F3106343.dat Mascot Search File - ../data/20190520/F3106344.dat Mascot Search File - ../data/20190521/F3106354.dat Mascot Search File - ../data/20190521/F3106356.dat

Acc. #	Protein ID	Pep. #	% Cov.	Score
13485	LO1-HUFAB_L0	n/a	67%	n/a
DIQMTQSPSSLSASVGDRTITCKASENVGIYVSWYQQKPGKAPKLLIYG ATKRYIGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCGQSYTYPYTFGG GTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCCLNNFYPREAKVQWKV DNALQSGNSQESVTEQDSKDESTYSLSSLTLSLKADYEKHKVYACEVTHQG LSSPVTKSFNRGE Red = MS-MS Underline = PMF Comments: Combined Sequence Coverage				

13485	LO1-HUFAB_L0	8	24%	70
DIQMTQSPSSLSASVGDRTITCKASENVGIYVSWYQQKPGKAPKLLIYG ATKRYIGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCGQSYTYPYTFGG GTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCCLNNFYPREAKVQWKV DNALQSGNSQESVTEQDSKDESTYSLSSLTLSLKADYEKHKVYACEVTHQG LSSPVTKSFNRGE Red = MS-MS Underline = PMF Comments: Individual ions scores > 28 indicate identity or extensive homology (p<0.05).				

Score	Peptide	Modifications	Obs. m/z	Delta	MS-MS?	PMF?
70	L.SASVGDRTITCKASENVGT.V		2,215.08	0.0111	☒	☐
0	L.SASVGDRTITCKASENVGTYSW.Y		2,587.26	0.0149	☐	☒
0	W.YQQKPGKAPKLL.I		1,370.86	0.0433	☐	☒
0	Y.QQKPGKAPKLL.L		1,094.64	-0.0329	☐	☒
0	Y.QQKPGKAPKLL.I		1,207.78	0.0299	☐	☒
0	Y.QQKPGKAPKLLIY.G		1,483.93	0.0344	☐	☒
0	Y.IGVPSRF.S		775.44	-0.0059	☐	☒
0	L.TLSKADY.E		797.42	0.0122	☐	☒

13485	LO1-HUFAB_L0	9	36%	81
DIQMTQSPSSLSASVGDRTITCKASENVGIYVSWYQQKPGKAPKLLIYG ATKRYIGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCGQSYTYPYTFGG GTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCCLNNFYPREAKVQWKV DNALQSGNSQESVTEQDSKDESTYSLSSLTLSLKADYEKHKVYACEVTHQG LSSPVTKSFNRGE Red = MS-MS Underline = PMF				

Comments: Individual ions scores > 21 indicate identity or extensive homology (p<0.05).

Score	Peptide	Modifications	Obs. m/z	Delta	MS-MS?	PMF?
0	-.DIQMTQSPSSLSASVGDR.V		1,878.91	0.0277	☐	☒
0	-.DIQMTQSPSSLSASVGDR.V	Oxidation (M)	1,894.90	0.0147	☐	☒
0	-.DIQMTQSPSSLSASVGDRVITITCK.A		2,581.26	0.0020	☐	☒
0	-.DIQMTQSPSSLSASVGDRVITITCK.A	Oxidation (M)	2,597.25	-0.0030	☐	☒
81	K.ASENVGTYVSWYQQKPGKAPK.L		2,338.19	0.0085	☒	☐
0	K.ASENVGTYVSWYQQKPGK.A		2,042.02	0.0174	☐	☒
0	K.RYIGVPSR.F		947.58	0.0420	☐	☒
0	R.YIGVPSR.F		791.47	0.0264	☐	☒
0	K.VYACEVTHQGLSSPVTKSFN RGE.-		2,566.16	-0.0768	☐	☒

☐ 13485	LO1-HUFAB_L0	17	39%	343
☐	DIQMTQSPSSLSASVGDRVTITCKASENVGIYVSWYQQKPGKAPKLLIYG ATKRYIGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCGQSYTYPYTFGG GTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCCLNNFYPREAKVQWKV DNALQSGNSQESVTEQDSKDESTYSLSSLTLSKADYEKHKVYACEVTHQG LSSPVTKSFN RGE			

Red = MS-MS Underline = PMF

Comments: Individual ions scores > 17 indicate identity or extensive homology (p<0.05).

Score	Peptide	Modifications	Obs. m/z	Delta	MS-MS?	PMF?
96	-.DIQMTQSPSSLSASVGDR.V		939.95	-0.0006	☒	☐
(70)	-.DIQMTQSPSSLSASVGDR.V	Oxidation (M)	947.94	0.0010	☒	☐
21	R.VTITCK.A		361.20	-0.0004	☒	☐
33	K.ASENVGTYVSWYQQKPGK.A		1,021.50	-0.0011	☒	☐
0	K.ASENVGTYVSWYQQKPGK.A		681.34	-3.0000	☐	☒
(23)	K.ASENVGTYVSWYQQKPGK.A		1,021.50	0.0020	☒	☐
38	K.LLIYGATK.R		439.77	-0.0009	☒	☐
38	K.LLIYGATKR.Y		517.82	-0.0004	☒	☐
(22)	K.LLIYGATK.R		439.77	0.0002	☒	☐
(27)	K.LLIYGATKR.Y		517.82	-0.0016	☒	☐
25	K.RYIGVPSR.F		474.27	-0.0002	☒	☐
34	R.YIGVPSR.F		396.22	0.0001	☒	☐
(32)	R.YIGVPSR.F		396.22	-0.0007	☒	☐
25	K.VEIKR.T		322.71	-0.0005	☒	☐
(20)	K.VEIKR.T		322.71	0.0003	☒	☐
(22)	K.VEIKR.T		322.71	-0.0003	☒	☐
33	K.VDNALQSGNSQESVTEQDSK.D		1,068.49	0.0015	☒	☐

☐ 13485	LO1-HUFAB_L0	48	44%	540
☐	DIQMTQSPSSLSASVGDRVTITCKASENVGIYVSWYQQKPGKAPKLLIYG ATKRYIGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCGQSYTYPYTFGG GTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCCLNNFYPREAKVQWKV DNALQSGNSQESVTEQDSKDESTYSLSSLTLSKADYEKHKVYACEVTHQG LSSPVTKSFN RGE			

Red = MS-MS Underline = PMF

Comments: Individual ions scores > 20 indicate identity or extensive homology (p<0.05).

Score	Peptide	Modifications	Obs. m/z	Delta	MS-MS?	PMF?
80	-DIQMTQSPSSL.S		603.79	-0.0008	☒	☐
66	-DIQMTQSPSSLSASVGDRVTITCKASENVGTY.V	Oxidation (M)	1,140.21	0.0005	☒	☐
(32)	-DIQMTQSPSSLSASVGDRVTITCKASENVGTY.V	Oxidation (M)	1,140.21	0.0016	☒	☐
(32)	-DIQMTQSPSSLSASVGDRVTITCKASENVGTY.V	Oxidation (M)	1,140.21	0.0052	☒	☐
(35)	-DIQMTQSPSSLSASVGDRVTITCKASENVGTY.V	Oxidation (M)	1,140.21	-0.0069	☒	☐
(40)	-DIQMTQSPSSLSASVGDRVTITCKASENVGTY.V	Oxidation (M)	1,140.21	0.0034	☒	☐
(47)	-DIQMTQSPSSLSASVGDRVTITCKASENVGTY.V	Oxidation (M)	1,140.21	0.0045	☒	☐
(53)	-DIQMTQSPSSLSASVGDRVTITCKASENVGTY.V	Oxidation (M)	1,140.21	0.0038	☒	☐
(58)	-DIQMTQSPSSLSASVGDRVTITCKASENVGTY.V	Oxidation (M)	1,140.21	0.0034	☒	☐
(68)	-DIQMTQSPSSL.S	Oxidation (M)	611.78	-0.0002	☒	☐
74	L.SASVGDRVTITCKASENVGTY.V		1,108.04	0.0003	☒	☐
(30)	L.SASVGDRVTITCKASENVGTY.V		739.03	-0.0012	☒	☐
(33)	L.SASVGDRVTITCKASENVGTY.V		739.03	-0.0014	☒	☐
(35)	L.SASVGDRVTITCKASENVGTY.V		739.03	0.0021	☒	☐
(36)	L.SASVGDRVTITCKASENVGTY.V		739.03	0.0034	☒	☐
(37)	L.SASVGDRVTITCKASENVGTY.V		739.03	0.0001	☒	☐
(38)	L.SASVGDRVTITCKASENVGTY.V		739.03	-0.0003	☒	☐
(39)	L.SASVGDRVTITCKASENVGTY.V		1,108.04	0.0005	☒	☐
(40)	L.SASVGDRVTITCKASENVGTY.V		739.03	0.0015	☒	☐
(40)	L.SASVGDRVTITCKASENVGTY.V		739.03	0.0023	☒	☐
(40)	L.SASVGDRVTITCKASENVGTY.V		739.03	0.0048	☒	☐
(41)	L.SASVGDRVTITCKASENVGTY.V		739.03	0.0004	☒	☐
(42)	L.SASVGDRVTITCKASENVGTY.V		739.03	-0.0003	☒	☐
(42)	L.SASVGDRVTITCKASENVGTY.V		739.03	0.0003	☒	☐
(42)	L.SASVGDRVTITCKASENVGTY.V		739.03	0.0019	☒	☐
(42)	L.SASVGDRVTITCKASENVGTY.V		739.03	0.0032	☒	☐
(46)	L.SASVGDRVTITCKASENVGTY.V		1,108.04	-0.0005	☒	☐
(46)	L.SASVGDRVTITCKASENVGTY.V		1,108.04	-0.0002	☒	☐
(46)	L.SASVGDRVTITCKASENVGTY.V		1,108.04	0.0012	☒	☐
(48)	L.SASVGDRVTITCKASENVGTY.V		1,108.04	0.0015	☒	☐
(51)	L.SASVGDRVTITCKASENVGTY.V		739.03	-0.0001	☒	☐
(51)	L.SASVGDRVTITCKASENVGTY.V		739.03	0.0012	☒	☐
(62)	L.SASVGDRVTITCKASENVGTY.V		1,108.04	0.0005	☒	☐
(67)	L.SASVGDRVTITCKASENVGTY.V		1,108.04	0.0034	☒	☐
39	W.YQKPGKAPKLL.I		685.91	0.0000	☒	☐
(22)	W.YQKPGKAPKLL.I		343.46	-0.0011	☒	☐
(25)	W.YQKPGKAPKLL.I		457.61	-0.0016	☒	☐
(32)	W.YQKPGKAPKLL.I		685.91	0.0002	☒	☐
(36)			685.91	-0.0003		

	W.YQKPGKAPKLL.I				☒	☐
46	Y.QQKPGKAPKLL.I		604.38	-0.0007	☒	☐
34	L.IYGATKRY.I		486.27	-0.0005	☒	☐
(32)	L.IYGATKRY.I		486.27	-0.0006	☒	☐
28	Y.GATKRY.I		348.20	-0.0003	☒	☐
(21)	Y.GATKRY.I		348.20	-0.0007	☒	☐
64	Y.IGVPSRFSGSGTDF.T		785.88	-0.0006	☒	☐
45	Y.IGVPSRF.S		388.23	-0.0012	☒	☐
41	F.TLTISLQPEDF.A		675.84	-0.0006	☒	☐
23	L.TLSKADYEKHKVY.A		527.95	-0.0006	☒	☐

Analysis Details

Supplemental Report	
Analyst Notes	LO1-HUFAB_L0 was identified.
Analysts	Borman, Satty [satty.k.borman@gsk.com]
Site	Stevenage UK
Instruments	Ultraflex Extreme (801613) - ABI - 4000 Qtrap OrbiTrap (501482B) - Thermo - LTQ Orbitrap

Sample / Submission Record

User Identity	
Scientist	Satty Borman [satty.k.borman@gsk.com]
Organization	Drug Design and Selection - DDS
Project	ID# 51659, CD96 pathway antagonist mAb (i.v.) - cancer
Submitted	17-May-2019
Completed	21-May-2019
Notebook #	N72965-1
Protein Deliverable / GRITS	n/a

Sample Information							
Sample ID	62680						
Name	LO1 Fab						
Buffer							
Notes							
Concentration							
Volume							
Injection Volume							
Protein Expr System							
Variable Modifications	<table><tr><th>Modification</th><th>MW</th></tr><tr><td>Acetylation</td><td>42.04</td></tr><tr><td>Loss of Methionine</td><td>-131.20</td></tr></table>	Modification	MW	Acetylation	42.04	Loss of Methionine	-131.20
	Modification	MW					
	Acetylation	42.04					
Loss of Methionine	-131.20						
Expected MW	25,640.85						
Batch							

Analysis Reports	
» Protein Identification ADV	
» Protein Identification ADV	
» Protein Identification ADV	

Gel	
Gel ID	14538
Gel File	Gel Editor: lo1.png file: lo1.png

Protein / MW				
Sequence ID		13484		
Sequence Name		LO1-huFab_H1		
Sequence MW		25,640.85		
Related / Additional Protein Sequences		LO1-huFab_L0 · MW = 23233.6963 · SeqID = 13485		
User Specified Phosphorylation Sites				
METDTLLLVV	LLLWVPGSTG	QVQLVQSGAE	VKKPGSSVKV	SCKASGYAFT
NYLIEWVRQA	PGQGLEWMGV	INPGSGGTNY	NEKFKGRVTI	TADKSTSTAY
MELSSLRSED	TAVYYCARSF	KWKFDYWGQG	TTVTVSSAST	KGPSVFPLAP
SSKSTSGGTA	ALGCLVKDYF	PEPVTVSWNS	GALTSGVHTF	PAVLQSSGLY
SLSSSVTVPS	SSLGTQTYIC	NVNHKPSNTK	VDKKVEPKSC	

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53	Table	Table 2.2: Imaging modalities stratified by nature of invasiveness at current stage of use.	Cardiovascular Imaging: Arterial and Aortic Valve Inflammation and Calcification	Springer International Publishing Switzerland	11.1.2021	Yes	Rightslink, agreement included
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